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UNIVERSITY OF ALBERTA

POLYMER-COATED LIPOSOMES. PREPARATION, STABILITY AND RELEASE OF ACETYLSALICYLIC ACID

BY

CUNJI DONG

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A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
PHARMACEUTICAL SCIENCES (PHARMACEUTICS)

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

EDMONTON, ALBERTA
SPRING 1992

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J. A. Rogers
DR. J. A. ROGERS (Supervisor)

Jamali
DR. F. JAMALI

RA Locock
DR. R. A. LOCOCK

TM Allen
DR. T. M. ALLEN

Michael Mezei
DR. M. MEZEI (External examiner)

DATE: Dec. 9 1991

DEDICATED TO

MY PARENTS

ABSTRACT

Three polymer systems, poly(4-vinylpyridine) (poly(4-VP)), carboxymethylchitin (CM-chitin), and acacia-gelatin, have been used to coat dimyristoylphosphatidylcholine (DMPC)/dicetylphosphate (DCP) or dipalmitoylphosphatidyl choline (DPPC) liposomes. The physical properties of polymer-coated liposomes, such as particle size, physical stability and acetylsalicylic acid (ASA) encapsulation and release, have been measured.

Negatively-charged DMPC/DCP liposomes reacted with the positive monomer, 4-vinylpyridine (4-VP), to form a salt and, subsequently, autopolymerized to form poly(4-VP)-coated liposomes. DPPC liposomes were coated with CM-chitin via the reverse-phase evaporation technique or were microencapsulated with acacia-gelatin by complex coacervation. The optimum concentrations of polymers were 8.5 mM 4-VP, 1% CM-chitin, or 3% acacia-gelatin. None of the polymer coatings affected the degree of ASA encapsulation.

A turbidity method for the quantitative determination of CM-chitin in liposome systems was established in the CM-chitin concentration range of 20 ~ 300 μg/ml. The method is simple, sensitive, and reproducible and is not influenced by the presence of liposome components such as egg phosphatidylcholine, DMPC, or DPPC.

The size of liposomes increased after polymer coating, except in the case of CM-chitin added during liposome formation which caused a decreased particle size, possibly through a polymer condensation effect. The average particle sizes varied depending on liposome composition and preparation method, being about 2 μm for poly(4-VP)-coated DMPC/DCP liposomes, 4 μm for CM-chitin-coated DPPC liposomes and 3.5 μm for acacia-gelatin-microencapsulated DPPC liposomes. Compared to control liposomes, the increased particle sizes due to polymer coating corresponded to a coating thickness of 0.17 μm of poly(4-VP)-coated liposomes and 0.39 μm of acacia-gelatin-microencapsulated liposomes, respectively.

The physical stability of poly(4-VP)-coated liposomes coated with an initial 4-VP concentration of 8.5 mM was significantly increased in the solutions of 8 mM sodium cholate or 16% aqueous isopropanol. Similarly, the physical stabilities of CM-chitin-coated liposomes and acacia-gelatin-microencapsulated liposomes in sodium cholate solutions were also increased. The increased stability of polymer-coated liposomes was in the order: CM-chitin > acacia-gelatin > poly(4-VP).

The release of ASA from polymer-coated liposomes decreased by 33% to 3-fold compared to uncoated liposomes at 37°C. The decreased release of ASA was in the order: CM-chitin > acacia-gelatin > poly(4-VP), which was the same order as found for the physical stability of liposomes.

Development of these formulations and their behavior *in vitro* suggest that the polymer-coated liposome might be effective as an oral drug delivery system.

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LIST OF ABBREVIATIONS

μ	ionic strength
4-VP	4-vinylpyridine
ASA	acetylsalicylic acid
CF	carboxyfluorescein
CHOL	cholesterol
CM-chitin	carboxymethyl chitin
DCP	dicetylphosphate
DMPC	dimyristoylphosphatidylcholine
DPPC	dipalmitoylphosphatidylcholine
DSC	differential scanning calorimetry
DSPC	distearoylphosphatidylcholine
EPC	egg phosphatidylcholine
ESR	electron spin resonance
GIT	gastrointestinal tract
GM_1	monosialoganglioside
HPLC	high performance liquid chromatography
kcps	thousand counts per second
Khz	kilo hertz
MLVs	multilamellar vesicles
NMR	nuclear magnetic resonance
p	probability

PC	phosphatidylcholine
PEG	polyethylene glycol
poly(4-VP)	poly(4-vinylpyridine)
PS	phosphatidylserine
PVP	polyvinylpyrrolidone
r	regression coefficient
RES	reticuloendothelial system
REV	reverse phase evaporation
SD	standard deviation
SUVs	small unilamellar vesicles
T_c	phase transition temperature
TEM	transmission electron microscope
TLC	thin layer chromatography
UV	ultraviolet

CHAPTER 1

INTRODUCTION

1.1. Liposomes — An Overview

Liposomes or lipid vesicles were first described by *Bangham et al. (1965)* as closed discrete structures capable of trapping water-soluble molecules. Liposomes were originally employed as a research tool in membrane science to investigate the properties of lipid bilayers, which were believed to be an essential structural element of biological membranes. Following studies on the characteristics and properties of liposomes, *Sessa and Weissmann (1970)* and *Gregoriadis et al. (1972)* considered using multilamellar vesicles as a drug delivery system for replacement therapy in the genetic deficiency of lysosomal enzymes. Since then, a wide variety of compounds have been successfully entrapped in liposomes and research on the use of liposomes as drug delivery systems has been a focus of the pharmaceutical industry.

A major advantage of the liposome as a drug delivery system is its biodegradable and, generally, nontoxic lipid components. The formulation of liposomes is very flexible and the physical properties obtained are usually a function of the nature and amount of the components used. Furthermore, since the phospholipids that form liposomes are often the same types which occur naturally in living cells, liposomes are able to interact with cell membranes, under the appropriate conditions, to deliver entrapped agents into cells.

Most research on liposomes as drug delivery systems has focused on intravenous administration, but some success has been obtained following ophthalmic, topical, or oral administration. Since the oral route is the most convenient, versatile, and patient-acceptable, it has long been hoped that liposomes would be useful in facilitating the absorption of drugs which are poorly-absorbed or unstable in the gastrointestinal tract (GIT) and, hence, increase the bioavailabilities of certain drugs. Until now, scientists have been unable to reach a consensus on the overall effect of liposome entrapment on the absorption of drugs and they still disagree on the feasibility of using liposomes as an oral drug delivery system. The main problem associated with orally-administered liposomes pertains to their relatively poor stabilities in the milieu of the GIT, due to the detergent effect of bile salts or the lytic effects of enzymes and, consequently, leakage of entrapped agents.

Many attempts have been made to increase the stability of liposomes. In the early stage of development, formulation work concentrated on the modification of liposome composition. For example, use of saturated lipids instead of unsaturated lipids or incorporation of the antioxidant α-tocopherol, yields liposomes of increased stability (*Hunt and Tsang, 1981*); incorporation of cholesterol (CHOL) into liposomes increased the stability of liposomes in serum (*Mayhew, 1979; Allen and Cleland, 1980; Kirby, 1980; Hunt, 1982*); use of longer chain of phospholipids, such as distearoylphosphatidylcholine (DSPC), can also increase the stability of liposomes (*Rowland and Woodley, 1980*). However, these types of liposomes apparently offer little improvement in liposome stability in the GIT, even for so called a "stable liposome" (DSPC/CHOL 2:1) (*Chiang, 1986*). More recently, polymerized and polymer-coated liposomes have been developed. It has been shown that polymerized liposomes *i.e.* liposomes in which the lipid molecules in the bilayers polymerize to form a continuum within the bilayer, greatly improved the stabilities of liposomes both *in vitro* and *in vivo*. However, the process of polymerization was sometimes not favourable for the stability of entrapped drugs, especially for inherently unstable drugs. Also, these ordered membranes were drastically different from original liposome structures or biological membranes. On the other hand, polymer-coated liposomes have proved to be more stable than uncoated liposomes in serum, and the ordered membrane-like structure was basically unchanged (*Sunamoto et al., 1983; Fukuda et al., 1986*). Therefore, it is possible that liposomes with suitable polymer coatings can be made more stable in the GIT than conventional liposomes.

1.2. Classification of Liposomes

Liposomes can be divided into three types according to their lamellar structure and size. They may occur as "onion-skinned" multilamellar vesicles (MLVs), large unilamellar vesicles (LUVs) or small unilamellar vesicles (SUVs). Since the introduction of polymerized vesicles and polymer-coated liposomes, a different classification of liposomes has evolved, referred to as *conventional liposomes*, *polymerized liposomes* and *polymer-coated liposomes*.

1.2.1. Conventional Liposomes

Conventional liposomes utilized as drug delivery systems typically range in diameter from 25 nm to several μm and include three categories, MLVs, LUVs and SUVs (*Kreuschner, 1986*). The structures of MLVs and LUVs can be depicted as in

Fig. 1.1. MLVs are made up of several lipid bilayers separated by water compartments and are heterogenous in size, ranging from 100 nm to 10 μm. LUVs consist of a single bilayer surrounding an aqueous core and sizes can range from 60 to 1000 nm. The structure of SUVs is essentially the same as LUVs except that the size of SUVs is much smaller, ranging in size from 20 to 50 nm.

1.2.2. Polymerized Liposomes

Polymerized liposomes can be classified according to direction in which the polymer backbone runs as shown in Fig. 1.2: a) through the centre of the hydrocarbon bilayer, b) through the lipid chains of inner and outer monolayers, c) through the polar head groups of each monolayer, d) through a monolayer lipid membrane or e) adjacent to the inner and outer surfaces of the bilayers (*Regen et al., 1984; Regen, 1987*).

1.2.3. Polymer-Coated Liposomes

There are basically four kinds of polymer-coated liposomes according to the mechanism of interaction between the polymer and the liposomal components (Fig. 1.3): (1) adsorption of polymer molecules at the liposome surfaces by hydrophilic or ionic force (*Seki and Tirrell, 1984*), (2) fixation of polymer molecules by hydrophobic anchor groups which penetrate the lipid bilayers (*Martin and Papahadjopoulos, 1982; Sunamoto et al., 1987*), (3) fixation of polymerizable, water-soluble molecules to the liposome surfaces via salt formation, followed by an initiation or spontaneous polymerization (*Aliev et al., 1984; Fukuda et al., 1986*), and (4) preparation of liposomes from amphiphiles which possess a polymerizable group linked to the polar

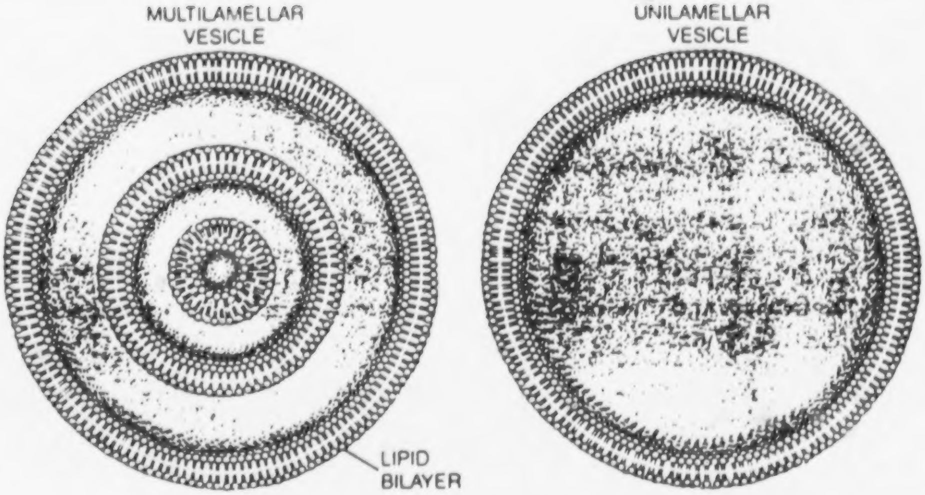
MULTILAMELLAR VESICLE
UNILAMELLAR VESICLE
LIPID BILAYER

Fig. 1.1. Schematic diagram of multilamellar (left) and unilamellar (right) liposomes.

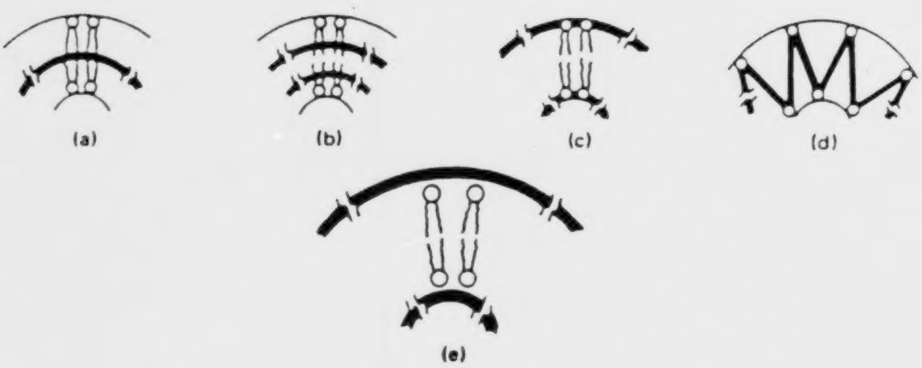

Fig. 1.2. Schematic illustration of possible mechanisms of polymerized liposomes (*Regen, 1987*).

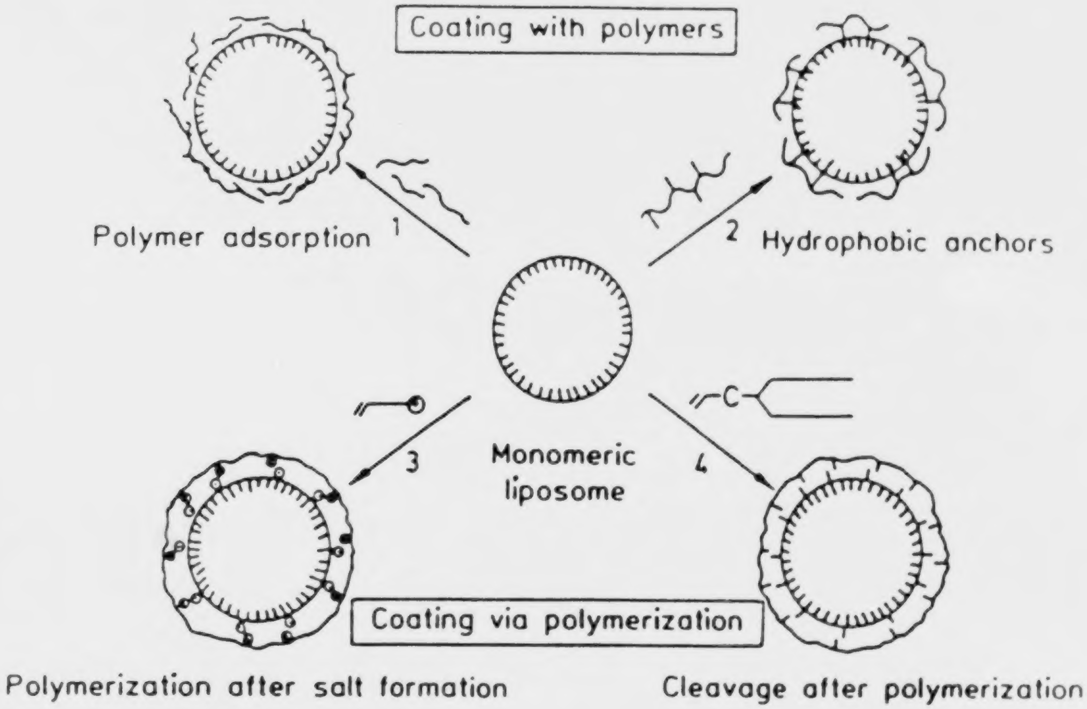
Coating with polymers
Polymer adsorption
1
2
Hydrophobic anchors
Monomeric liposome
3
4
C
Coating via polymerization
Polymerization after salt formation
Cleavage after polymerization

Fig. 1.3. Schematic illustration of possible ways to create stabilized liposomes by surface coating with polymer (*Aliev et al., 1984*).

head group by a cleavable spacer and cleavage of the spacer after polymerization. The application of category (4) has not been adequately demonstrated to date.

1.3. Methods of Preparing Liposomes

Liposomes can be prepared from a variety of lipids and lipid mixtures (*Gregoriadis, 1976; Szoka and Papahadjopoulos, 1980*). Phospholipids are most often used, but vesicles can also be prepared from lysophosphatides in the presence of equimolar CHOL (*Kitagawa et al., 1976*) or from other single chain amphiphiles (*Gebicki and Hicks, 1973, 1976; Tall and Small, 1977; Hargreaves and Deamer, 1978*).

There are variety of methods for preparing liposomes. The ideal preparation method should be one that is able to produce liposomes from a variety of lipid components over a broad range of lipid concentrations and having a relatively homogeneous size distribution. Finally, the method should be rapid, efficient and not damage or contaminate the lipid components.

1.3.1. Preparation of Conventional Liposomes

1.3.1.1. Multilamellar vesicles (MLVs)

The method of preparation of multilamellar vesicles was first introduced by *Bangham et al. (1965)*, and hence, is often referred to as the *Bangham method*. In this method, the lipids are dissolved in an organic solvent (*e.g.* chloroform) in a round-bottom flask and, subsequently, a thin lipid film is formed on the inside wall of the flask after removal of the organic solvent by rotary evaporation under reduced pressure.

Finally, water or aqueous buffer is added and the dried film becomes hydrated, most efficiently if mixed at a temperature above the phase transition temperature (T_c) of the constituent phospholipids. This can be accomplished by gentle hand-shaking and/or vortex-mixing to yield a dispersion of MLVs. The advantages of the *Bangham method* are simplicity of preparation and ready entrapment of a large variety of substances. The major drawback of MLVs is their heterogeneity with respect to particle size and number of lamellae of the liposomes, although with hydrophilic solutes they encapsulate greater amounts than SUVs.

1.3.1.2. Large unilamellar vesicles (LUVs)

Large unilamellar vesicles are usually prepared by the reverse phase evaporation (REV) method (*Szoka and Papahadjopoulos, 1978*). The lipids are dissolved in a volatile organic solvent, then the aqueous phase is added to the organic phase and the mixture is sonicated to form an emulsion followed by slow removal of the organic phase via rotary evaporation under reduced pressure. Vesicles prepared by this technique have a high aqueous space-to-lipid ratio which can be advantages for incorporation of water-soluble agents. The principal disadvantage of this method is the exposure of the drug to organic solvents and to short periods of sonication, which, for example, may denature proteins.

In addition to the REV method, the solvent injection method (*Deamer and Bangham, 1976*), the extrusion method (*Hope et al., 1985*), the fusion method (*Papahajopoulos et al., 1975*), the pH adjustment method (*Hauser and Gains, 1982*), or the formation of liposomes from template surfaces (*Lasic et al., 1988*) have been advocated for the preparation of LUVs.

1.3.1.3. Small unilamellar vesicles (SUVs)

Sonication of MLVs above the T_c of the lipids under an inert atmosphere results in the formation of SUVs (*Saunders et al., 1962*). During sonication the structure of MLVs is broken down and SUVs with high radius of curvature are formed. The major advantage of SUVs as drug delivery systems has been their decreased systemic clearance. However, their use is drastically limited because of a low encapsulation efficiency, a size limitation of solute that can be encapsulated (*e.g.* not macromolecules) and asymmetric distribution of the various lipids used between the inner and outer monolayers.

Other methods used in the preparation of SUVs are detergent removal (*Kagawa and Racker, 1971*), microfluidizer (*Mayhew et al., 1984*), and French pressure cell (*Hamilton and Guo, 1984*).

1.3.2. Preparation of Polymerized Liposomes

The most common strategy used in preparing polymerized vesicles is first to assemble the vesicles from suitable polymerizable lipids and then to carry out polymerization. An alternative approach, which has received little attention thus far, is based on the prior formation of a lipid polymer and subsequent vesicle assembly. The position of polymerization in lipid depends on the location of unsaturated bonds as well as the method used to catalyze polymerization (*Regen, 1987*).

1.3.2.1. Preparation from polymerizable lipids

In essence, polymerized vesicles can be prepared by applying all of the available existing conventional liposome techniques by using polymerizable lipids with a wide assortment of sizes and lamellar states. Polymerization is then catalyzed by ultraviolet (UV) light or some free-radical initiators. UV catalysis is the simplest and most commonly used method, *e.g.* lipids containing conjugated diacetylene, conjugated diene, methacrylate, and thiol groups can be polymerized by this method (*Day and Ringsdorf, 1978*). MLVs and LUVs polymerize effectively below the T_c of lipids upon brief exposure to UV irradiation. SUVs have high surface curvature, and hence, showed substantially reduced photopolymerizability (*Lopez et al., 1982*). Another method used to promote polymerization of polymerizable lipids includes condensation polymerization with the aid of standard free-radical initiators, such as azobisisobutyronitrile (*Ohno et al, 1987*) or hydrogen peroxide (*Regen et al., 1983; Samuel et al., 1985*). The major advantages of this method, in principle, are that a high-molecular-weight and even cross-linked polymer networks can be obtained and the polymerized vesicles can be assembled in a controlled manner. The disadvantages are that the resulting polymers are not pure and the conditions for polymerization may lead to the degradation of other vesicular components (*e.g.*, entrapped drugs, membrane-bound proteins).

1.3.2.2. Preparation from polymerized lipids

In the preparation of polymeric liposomes, polymerizable lipid is first polymerized by polycondensation to form polymerized lipid. Subsequently, polymerized lipid is sonicated to form liposomes via side chain aggregation (*Kunitake et al., 1981 & 1984; Elbert et al, 1985; Neumann and Ringsdorf, 1986*). The advantage of this method is that impurities are not trapped in the liposomes since the polymerization occurred before assembly. Polymerized lipid can be purified by conventional solubilization-precipitation procedures. The disadvantage is that only relatively low molecular weight polymer is able to form liposomes which is unlikely to improve liposome stability significantly.

1.3.3. Preparation of Polymer-Coated Liposomes

The most frequently used technique in preparing polymer-coated liposomes is to incubate the liposome dispersion with the polymer solution. By specific or nonspecific interactions between polymers and lipid vesicles, the polymer is able to deposit on the surface of liposomes with or without hydrophobic anchor in the bilayer. For the non-specific interaction between liposomes and polymers, a hydrophobic anchor in the polymer is necessary in order to prepare stable polymer-coated liposomes. Two alternative approaches which are described in this thesis to increase the non-specific interaction between polymers and liposomes are: 1) adding the polymer during the liposome formation or 2) the microencapsulation of liposomes with polymers using a coacervation technique.

1.3.3.1. Preparation of polymer-coated liposomes via salt formation

Charged liposomes are first prepared in the same manner as conventional liposomes then oppositely-charged monomers are bound to liposome surfaces via salt formation. The monomers subsequently undergo spontaneous polymerization under the set conditions and over a period of time. Usually, the negatively-charged liposomes are formed with the addition of dicetylphosphate (DCP), phosphatidic acid, dihexadecylphosphate, or phosphatidylserine (PS). 4-vinylpyridine and choline methacrylate have been used as positively charged monomers. The advantages of this approach are that the preparation of polymer-coated liposomes is relatively easy and the kinetics of the polymerization process, for some compounds, can be followed by UV spectral analysis. A possible disadvantage of this approach is the inconvenience of removing the unreacted monomers before the preparation could be used clinically because of possible toxicities (*Aliev et al., 1984; Ringsdorf and Schlarb, 1988*).

1.3.3.2. Preparation of polymer-coated liposomes bearing hydrophobic anchor

Polymers bearing hydrophobic chains are synthesized before carrying out the coating procedure. Polymers which have been used include dextran, pullulan, amylopectin, and poly(carboxylic acid)s to which a palmitoyl or cholesterol group has been attached as the hydrophobic anchor (*Arnold et al., 1986; Takada et al., 1984; Sunamoto et al., 1987 & 1988; Sato, 1990*). The method of preparing liposomes is essentially the same as that for conventional liposomes then these are incubated with polymer solution either above or below the T_c of the lipid used to prepare the liposomes. Finally, the free polymer can be separated from the bound polymer by gel-filtration using a Sepharose 4B column. The advantage of this method is ease of preparation. In contrast, polymer without a hydrophobic anchor does not interact sufficiently strongly with liposomes to yield a stable polymer coating (*Sunamoto and*

Iwamoto, 1985).

1.3.3.3. Preparation of polymer-coated liposomes via hydrophilic adsorption including microencapsulation

Liposomes coated with polymers via hydrophilic adsorption are prepared by incubation of polymer solution with preformed liposomes. In order to increase the interactions between polymers and liposomes, the reverse phase-evaporation method has been used to deposit the polymer at the surfaces of liposomes during their formation. Lipids are dissolved in a volatile organic solvent and aqueous buffer is added in the same manner as the REV method of preparing conventional liposomes. After sonication above the T_c of the lipid, aqueous solution of polymer is added to the W/O emulsion and the mixture is vortex-mixed to form a W/O/W multiple emulsion. Polymer-coated liposomes are formed during the evaporation of the organic phase under reduced pressure. In this way, the non-specific interaction between polymers and liposomes is increased. Carboxymethylchitin (CM-chitin) has been successfully used to coat liposomes by this technique (*Kato et al., 1984; Izawa et al., 1986; Kato and Kondo, 1987*).

Another approach in the use of polymers with liposomes is to use the microencapsulation technique. For example, using the approach of complex coacervation by acacia-gelatin, liposomes are mixed with acacia-gelatin solution at 40°C which is then emulsified in an organic solvent to form a W/O emulsion. The temperature of the emulsion is adjusted to 10°C and the formed microcapsules are hardened with formaldehyde. The advantage of this method is the ability to successfully coat liposomes with polymers which do not have a specific functional group (*Yeung and Nixon, 1988; Nixon and Yeung, 1989*). A possible disadvantage is the involvement of organic solvent which could be detrimental to the liposomes.

1.4. Physical Characterization of Liposomes

1.4.1. Phase Transition Temperature

An important physical-chemical parameter of liposomes is that their constituent phospholipids undergo a sharp order-to-disorder transition at a characteristic temperature. The temperature at which the lipid state transforms from a condensed gel-like state to a fluid, liquid-crystalline state is called the phase transition temperature (T_c). The T_c is a function of the nature of the lipids and the surrounding environment. This temperature has been determined using a wide range of physical techniques, such as, X-ray diffraction (*Caffrey, 1989*), differential scanning calorimetry (DSC) (*Lewis and McElhaney, 1985*), dilatometry (*Cevc and Marsh, 1987*), NMR spectrometry (*Lewis and McElhaney, 1985*), ESR spectroscopy (*Marsh and Watts, 1981*). Among these, DSC is the most frequently used method because of its sensitivity and simplicity.

The T_c of phospholipid is dependent partly on the *van der Waals* forces between hydrocarbon moieties of the molecules, interactions between polar groups, and extent of hydration. In general, in a homologous series of phosphatidylcholines the T_c increases as the fatty acid chain length increases; introducing *cis* double bonds or branched chains tends to lower the T_c. A phospholipid with a head group of ethanol-amine has a T_c about 20°C higher than that of a phosphatidylcholine (*Juliano, 1983*).

When phospholipids having different T_cs are mixed, a modified T_c is obtained. A mixture of homogenous saturated phospholipids, differing by only two carbons in the fatty acid chain length will normally display a broad transition. On the other hand, when two very different phospholipids are mixed, two separate transitions representing the individual components are observed (*Shimshick and McConnell, 1973*). The addition of CHOL usually broadens the T_c. At temperature below the T_c, CHOL tends to expand and fluidize the bilayer, while at temperatures above the T_c, CHOL tends to increase the packing and rigidity of the bilayer (*Demel and DeKruyff, 1976*).

Different preparations of phospholipid liposomes may show different T_c values. MLVs and LUVs have very nearly the same T_c, but SUVs exhibit a somewhat lower T_c and broader transition. This is due to the smaller radius of curvature of SUVs which are in a somewhat strained metastable state (*Lichtenberg et al., 1975; Mendelsohn et al., 1976*).

1.4.2. Particle Size and Size Distribution

The size distribution of lipid vesicles is often a crucial characteristic in determining the significance of biochemical, biophysical or drug delivery experiments with liposomes. Many properties, such as stability, absorption, distribution and clearance can be influenced by liposome size and size distribution.

Many techniques have been used to characterize the mean particle size and particle size distribution of liposomes. Light microscopy is convenient to use for examining the size distribution of large vesicles and even particle shape but it is unsuitable for resolving the size of small liposomes (*Nassander et al., 1990*). For small liposomes, electron microscopy can be applied but the presence of some large vesicles may become disrupted during preparation of the sample and lead to erroneous results. However, by using both light and electron microscopy, a complete picture of the size distribution can be obtained. Light scattering is a non-destructive analytical method for sizing small particles and can be applied to follow changes in size as a function of time. However, dust particles which are present in the solution or dispersion can lead to errors in measurements of the average diameter (*Ruf et al., 1989*).

1.4.3. Encapsulation Efficiency of Drugs

Encapsulation efficiency refers to the fraction of solute sequestered by liposomes and usually is expressed either as percentage entrapment of total solute or amount of solute entrapped per mole of lipid. It is a function of the hydrophilicity of the drug, the lipid composition, and the method of preparation. Polar or hydrophilic drugs are encapsulated in the internal aqueous compartments of liposomes, whereas hydrophobic or amphiphilic drugs can partition into the liposome membrane (*i.e.* the bilayer) which generally yields greater encapsulation (*Stamp and Juliano, 1979*). Since non-polar drugs reside in the membrane environment, the encapsulation in fluid membranes is often higher than in rigid membranes. Since polar drugs are encapsulated in the polar region of liposomes, their encapsulation will depend on the total aqueous volume sequestered by the liposomes. The partitioning of a lipid-soluble drug in a bilayer can affect the partitioning of another lipid-soluble solute whereas the encapsulation of polar compounds does not usually influence the encapsulation of other solutes. *Szoka and Papahadjopoulas (1978)* have found an encapsulation efficiency of 0.5 ~ 1.0% for

SUVs, 5 ~ 15% for MLVs and 35 ~ 65% for LUVs respectively. Once a drug of low molecular weight is entrapped, its retention within the liposome is primarily determined by the bilayer permeability, which depends mainly on the size and charge of the drug and on the physical status of the membrane (*Yatvin and Lelkes, 1982*).

1.4.4. Stability

The stability of liposomes *in vitro* pertains to aspects of structural integrity during the storage period, minimization of oxidation products, and retention of entrapped agents. *In vivo* it pertains to the resistance against disruption by serum proteins, enzymes, bile salts, and pH changes. Liposome stability is usually evaluated by monitoring the leakage of an entrapped marker and by their observed physical integrity. The process of leakage of entrapped marker will be discussed in the section on drug release. This section discusses only the physical integrity of liposomes. One method of testing the stability is by mixing the liposomes with detergent solutions and monitoring the turbidity changes spectrophotometrically at either 400 nm (*Regen et al., 1980*) or 550 nm (*Defrise-Quertain et al., 1985*). The degree of turbidity reflects the extent to which liposome integrity is maintained.

Liposomes are energetically stable since they represent the most favourable thermodynamic state of the phospholipids in water (*Juliano and Layton, 1980*). However, liposomes represent a heterogenous system with potential chemical and physical problems (*Frokjaer et al., 1984*). Chemically, phospholipids are susceptible to hydrolysis and, in addition, unsaturated phospholipids can also undergo oxidation. Usually, under controlled conditions, the reactions of oxidation and acid/base catalyzed ester hydrolysis do not pose significant stability problems (*Hunt and Tsang, 1981*). In a physical sense, the stability of liposomes is a function of the lipid composition and the method of preparation. With regards to lipid composition, a significant increase in liposome stability in serum can be obtained after incorporation of CHOL. MLVs are usually more stable than unilamellar liposomes due to their larger number of bilayers. Sonicated SUVs are the least stable and these tend to aggregate and fuse together when below their T_c because of being in a metastable state (*Wong and Thompson, 1982*).

Polymerized liposomes differ substantially from conventional liposomes in terms of their stability toward mechanical and chemical perturbation. Remarkably, they retain their overall physical integrity even after mild ultrasonication and exposure to organic solvents and detergents. In addition, they have a greater resistance to osmotic shock. Furthermore, it has been possible to obtain scanning electron micrographs of polymerized liposomes whereas with conventional liposomes this is not easily done; the bilayer structure remains intact in 50% ethanol, and the leakage rate of entrapped substances can be greatly reduced (*Hupfer et al., 1983; Juliano et al., 1984*).

Applying polymer coatings is another approach that can be used to stabilize liposomes. Liposomes coated with derivatives of polysaccharides are stable enough to resist enzymatic lysis, while conventional liposome are destroyed under the same conditions (*Sunamoto et al., 1983*). Also, CM-chitin-coated liposomes have been demonstrated to be more resistant to detergents (*Kato and Kondo, 1987*). These indications suggest that polymer-coated liposomes have the potential to be used successfully by the oral route. This aspect will be discussed further in the section on liposome stability in the GIT.

1.4.5. Drug Release

In order for liposomes to be useful as drug delivery vehicles, the characteristics of drug release must be well-understood. Drug release from liposomes is a direct function of their integrity and permeability. The integrity of liposomes (stability) has previously been discussed, whereas this section only discusses the drug release as a function of liposome permeability.

The release of solute from a liposome preparation may be evaluated using several techniques. Dialysis has been a popular method used to follow drug release from liposomes. However, unless the release is much faster through the dialysis membrane than from liposomes, calculation of true release is complicated even in the modified dialysis method (*Sad et al., 1983*). Another method involves the incubation of the liposome preparation at a fixed temperature followed by separation of the released drug from entrapped drug via ultracentrifugation (*Magin and Niesman, 1984*) or gel filtration (*Finkelstein and Weissmann, 1979*). Both methods can directly yield information about the amount of released drug. A limitation of ultracentrifugation is the inability to sediment SUVs or very small MLVs. Gel filtration results in a large dilution of the sample, which may yield an analytical problem unless the detecting method is very sensitive, *e.g.* quenched fluorescent compounds or radioisotope-labelled compounds.

The rate of drug release from liposomes is a complex function of hydrophilicity of the drugs, the temperature, and the liposome composition. Generally, higher release rates are reported for hydrophobic drugs than for hydrophilic drugs. Rates of release increase with increase of the incubation temperature, and there is a sharp increase in the rate of drug release near the transition temperature of the lipid. Also, a decrease in chain length or an increase in unsaturation of the phospholipid generally leads to increased drug release from liposomes (*De Gier et al., 1968*). The incorporation of sphingomyelin and cholesterol are known to considerably decrease the rate of release.

Polymerized and polymer-coated liposomes yield greatly decreased drug release compared to conventional liposomes (*Sunamoto et al., 1984; Regen, 1985*), however, the total influence of polymer on the release of drug has not been fully characterized. Further work needs to be done to prove that these polymer-stabilized liposomes will be useful in controlled drug delivery.

1.5. Liposomes as a Drug Delivery System

The use of liposomes as a drug delivery system is recognized as a promising method of improving drug selectivity (*Gregoriadis, 1977*). There have been numerous reports on a variety of therapeutic agents carried by liposomes, such as, anticancer agents (*Ogihara-Umeda and Kojima, 1989; Mayer et al., 1990; Gabizon et al., 1990; Matzku et al., 1990*), antifungal drugs (*Lopez-Berestein et al., 1985; Heymans et al., 1990*), antibiotics (*Perez-Soler and Priebe, 1990; Price et al., 1990*), antiviral drugs (*Kim et al., 1990*), antiparasitics (*Alving et al., 1980 & 1984*) and other agents which could benefit from liposome delivery (*McCalden and Levy, 1990; Kleinerman and Jaffe, 1990*). In order to effectively deliver drug to specific targets, it is necessary to thoroughly study the mechanisms of liposome uptake and the fate of liposomes *in vivo*.

1.5.1. *In vitro* Studies

One of the most important features of liposomes as effective carriers is to deliver drug into target cells. Basically, there are four possible pathways by which liposomes may interact with cells: stable adsorption, endocytosis, fusion and lipid exchange (Fig. 1.4). (*Pagano and Weinstein, 1978*). Although each pathway is distinctly different from the other, multiple pathways of uptake might occur in different situations.

Stable adsorption is the association of intact vesicles with cell surfaces, where the vesicles are not able to be removed from the cell surface by repeated washings with buffer solution. This mechanism could be mediated by non-specific electrostatic, hydrophobic or other forces, or by specific interactions of the liposomes with components present at the cell surfaces. Demonstration of this type of adsorption is dependent on the incubation conditions as well as on the lipid composition. Neutral liposomes and vesicles containing beef brain sphingomyelin are very prone to cell surface adsorption at temperatures below the T_c of the incorporated lipids. Positively-charged liposomes have relatively strong affinities for cell surfaces even at the temperatures above the T_c of the lipids. Cell surface adsorption of liposomes can be reversed by treatment of the cells with trypsin suggesting a role of cell surface proteins in this process (*Pagano and Takeichi, 1977*). After adsorption the liposomal contents leaking out can be transported into the cell.

Endocytosis is another mechanism by which intact vesicles may be carried into cells after which the liposomes usually appear in the cell lysosomes. This process is dependent on the liposome composition and type of cells encountered. For cells that are

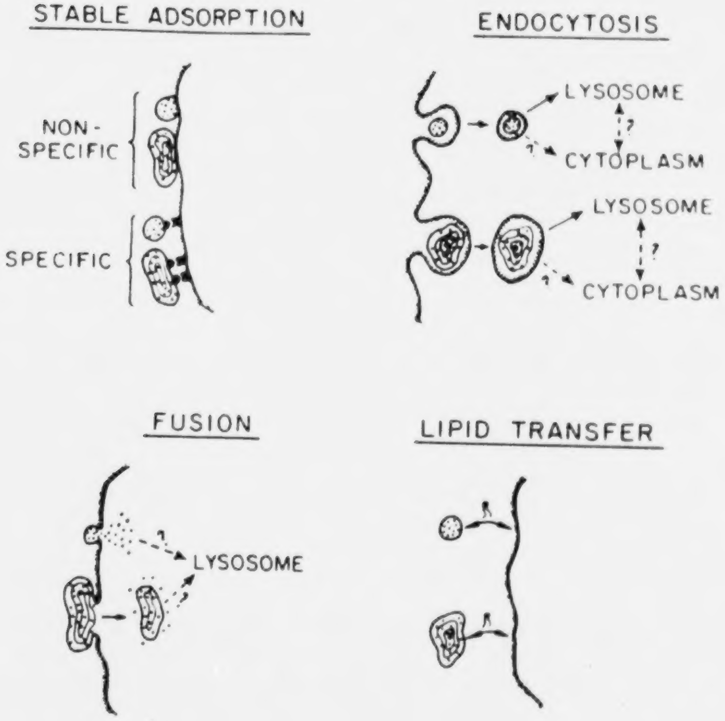
STABLE ADSORPTION
NON-SPECIFIC
SPECIFIC
ENDOCYTOSIS
LYSOSOME
?
CYTOPLASM
LYSOSOME
?
CYTOPLASM
FUSION
LYSOSOME
LIPID TRANSFER

Fig. 1.4. Mechanisms of interaction between liposomes and cells. Possibilities for both multilamellar and unilamellar vesicles are shown (*Pagano and Weinstein, 1978*).

less endocytotic than macrophages, the type of liposomes can play a major role in the endocytotic process. Although some data indicate that endocytosis is more likely to occur for gel-state liposomes (*Papahadjopoulos et al., 1975*), endocytosis of both fluid and gel-state SUVs and MLVs has been reported (*Pagano and Weinstein, 1978*). This mechanism of uptake has also been reported to increase the incorporation of liposomes in everted rat intestinal sac studies (*Rowland and Woodley, 1981*), which has generated further interest in developing orally-administered liposomes.

A third mechanism by which liposomes are thought to interact with cell membranes is by fusion in which the vesicle bilayer merges with the plasma membrane bilayer followed by release of the vesicle contents into the cytoplasmic space. *Poste et al. (1982*) suggested that the fusion mechanism for the uptake of LUVs is advantageous pharmacologically since encapsulated intracellular active drugs could be delivered directly into the cytoplasm. In this process, vesicle uptake is little affected by inhibitors of endocytosis. However, the liposome-cell interaction was found to be significantly influenced by the charge and fluidity of liposomes (*Poste and Papahad-jopoulos, 1976*). A case in point is with regards to negatively-charged, fluid liposomes which have been shown to be incorporated into cells mainly by a fusion process.

Lipid exchange between vesicles and the cell surfaces may also modulate the transfer of entrapped agents into cells. This mechanism does not require cellular uptake of intact vesicles and does not involve a net mass transfer of phospholipid between vesicles and cells, but soluble phospholipid exchange is catalyzed by associated proteins. It would appear that this pathway has little significance in the drug delivery process even though it might play a major role to serve lipid transport between cells and tissues.

1.5.2. *In vivo* Studies

Studies of liposome interactions with cells *in vitro* have offered some expectations of the possibility of obtaining the "magic bullet" in drug delivery. Specific targeting attempts and the development of sustained release formulations of liposomes have attracted considerable interest in applying liposomes in drug delivery to man and to laboratory animals by various routes of administrations including intravenous, intraperitoneal, intramuscular, subcutaneous, topical, and oral.

Studies involving the targeting of liposomes to tissues or cells have been mainly carried out by the intravenous and intraperitoneal routes. Initially, it was generally assumed that liposomes would not be recognized as a particulate and would thus avoid the reticuloendothelial system (RES). Hence, attachment of a variety of site-specific molecules, such as antibodies on the liposome surface, would have the potential for targeted liposomal drug delivery. Unfortunately, regardless of the composition, size, or charge of the vesicle and regardless of what targeting agent was used, liposomes are extensively extracted by the liver and spleen, the primary sites of phagocytic cells of the RES. An additional problem involves the inability of liposomes to leave the circulatory system and enter the extravascular space.

Naturally, the retention and uptake of circulating liposomes by the RES is a major advantage for liposomes being targeted to the RES organs. This has been successfully used in the treatment of *Leishmaniasis* infection (*Croft, 1986*) and systemic fungal infections (*Sculier et al., 1988*). Liposome encapsulation of meglumine antimoniate resulted in more than a 700-fold increase in its therapeutic efficacy as antileishmaniasis agent compared to the free drug (*Alving et al., 1978*). Also, liposomal amphotericin B has been advanced to clinical trials for improved efficacy in the treatment of systemic infections of cancer patients by two groups (*Sculier et al., 1988; Lopez-Berestein, 1988 & 1989*) and has been used in cancer patients with systemic fungal infections. The results showed that a liposomal product was more effective and reduced the toxicity of these drugs, although, the mechanisms involved are not yet fully understood.

Unfortunately, the uptake of liposomes by the RES is a major obstacle for targeting to other tissues and organs. Several approaches have been used to induce significant redistribution of *i.v.* administered liposomes away from RES cells to other tissue sites. One of the early approaches was to block the phagocytotic uptake by pre-dosing with empty liposomes or other inert particles. But this blockage of the host RES impairs the body's defences against pathogenic microorganisms, parasites and tumour cells, host responses to endotoxin and haemorrhagic shock, and responses to circulating immune complexes, which could lead, for example, to increased susceptibility to infection, or to the increased metastatic spread of tumours (*Allen, 1988*). Alternatively, liposome stability in plasma may be improved by the inclusion of CHOL, for example, which also exerts a significant effect on the rate of liposome clearance, *i.e.* a markedly increase in $t_{1/2}$. However, this did not alter the common tissue disposition patterns, for example, to the liver and spleen (*Senior, 1987*).

Recently, it was found that inclusion of monosialoganglioside (GM_1), phosphatidylinositol, sulfatides, or sphingomyelin dramatically decreased the uptake of liposomes by the RES and prolonged their circulation times (*Allen and Chonn, 1987;*

Allen, 1989; Papahadjopoulos and Gabizon, 1990). As a result, the increase in tumor uptake of such a liposome formulation was about 25-fold compared to conventional liposomes. The phenomenon of prolonged circulation and reduced RES uptake might be related to a decreased opsonization, probably obtained by a mimicking of the important properties of the outer monolayer of red blood cells. In spite of all these advances in the successful improvement of liposome circulation half-lives, there still remains the problem of liposomes being unable to leave the circulatory system and enter the extravascular space, which precludes targeting to most sites because the biological barriers separating the liposome from its ultimate site of action in the cell is still formidable.

Site-specific targeting of liposomes, on the other hand, may be advantageous because of the ready accessibility of certain organs such as the skin, eyes, lungs and body cavities. In this regard, liposome-mediated drug delivery is relatively easy to achieve by enhancing local and minimizing systemic effects (*Mezei, 1988*). As a drug carrier for topical administration, the liposomes act as a localizer rather than a transporter. In contrast to *i.v.* administration the retention of liposomes at the topical site of administration is an advantage in local drug therapy. The successful use of liposomes in topical drug delivery includes, 1) increased drug concentration in the epidermis and dermis and decreased percutaneous absorption (*Mezei, 1988*); 2) prolonged retention of drug in the cornea (*McCalden and Levy, 1990*); 3) enhanced lung-accumulation of liposomes for the treatment of lung tumors by inhalation (*Mihalko et al., 1988*); and 4) localized drug in the peritoneal cavity after intraperitoneal injection for the delivery of chemotherapeutic drugs to tumours (*Kim et al., 1987*).

As an oral drug delivery system, some apparent success has been obtained with liposomes, but the data are not convincing or consistent. Detailed discussions, including the efficiency of absorption after the oral administration of liposomes, some important mechanisms, the stability of liposomes in the GIT, and *in vitro* and *in vivo* uptake of liposomes, will be discussed in the following section.

1.6. Liposomes for Oral Administration of Drugs

The advantages of the oral route of administration of drugs are convenience, versatility and safety. However, some drugs can not be given orally due to their chemical instability or poor absorption from the GIT. Liposomes have been found to offer some degree of protection against degradation of labile drugs (*D'Silva and Notari, 1982; Habib and Rogers, 1987*). In spite of these *in vitro* results, it has been the experience of most researchers that liposomes were not able to maintain their integrity under physiological conditions in the GIT. Nevertheless, liposomes have been reported to increase the oral bioavailibility of many drugs (*Hashimoto and Kawada, 1979; Patel et al., 1982; Woodley, 1985; Petkowicz et al., 1989*).

In order to be suitable for oral use, liposomes should be easy to prepare, have satisfactory encapsulation efficiencies for drugs, be stable in the GIT, be nontoxic, and promote absorption of their entrapped agents.

1.6.1. Toxicity of Oral Liposomes

Since most liposome formulations are composed of naturally-occurring phospholipids, which would be present in the normal diet, oral toxicities are unlikely to be a problem. Toxicity may be more of a problem if liposomes become absorbed into the general circulation, especially those containing stearylamine. However, the quantities of lipid absorbed after oral administration of liposomes are likely to be very small. The toxicity of polymer-coated liposomes depends on the toxicity of the polymers used. Most polymers, such as CM-chitin, acacia-gelatin, have often been used in pharmaceutical dosage forms and are considered to be safe, inert materials. However, the toxicity of some polymers, such as poly(4-VP), is unknown and may be potentially toxic.

1.6.2. Stability of Liposomes in the GIT

Liposomes have been shown to be unstable and be susceptible to destruction by bile salts and lipases, or simply by changes in pH which occur in the GIT. There are varying reports on this subject. For instance, liposomes exposed to simulated gastric fluid in acidic media at pH 1.2 in the presence of pepsin were found to be fairly stable (*Hashimoto and Kawada, 1979*). Others also agree that liposomes of the appropriate composition can protect most of their contents from degradation during passage through the stomach (*Patel and Ryman, 1977; Rowland and Woodley, 1980; Chiang, 1986*).

On the other hand, *Love et al. (1990)* found that the liposomes they studied were very unstable in simulated intestinal fluid composed of phosphate buffer, bile salt, and pancreatic lipase. The stability of liposomes against pancreatic phospholipase A_2 is reported to be a function of the T_c of the phospholipid. Interestingly, both at temperatures above or below the T_c, phospholipase A_2 had little effect on the integrity of the liposomes whereas at temperatures close to the T_c, enzymatic hydrolysis of the lipids occurred because the bilayers are disorganized, highly fluid and permeable to ions and small molecules (*Op Den Kamp et al., 1974; Richards and Gardner, 1978*).

Generally, fluid liposomes are more susceptible to disruption than gel-state liposomes. Thus, liposomes composed of DSPC/CHOL at 7:2 molar ratio and DPPE/CHOL/DCP at 7:2:1 molar ratio, which are in the gel state at physiological temperature, were found to be relatively more stable than other liposomes even in bile salt solutions (*Rowland and Woodly, 1980*), but they were still not sufficiently stable to survive in intestine fluid.

Both polymerized and polymer-coated liposomes have been found to be more stable than conventional liposomes. However, few studies have been carried out with respect to their stabilities in the GIT, except one negative result by *O'Connor et al. (1985)*. These authors showed that the observed increased stability of egg phosphatidylcholine liposomes with branched polysaccharide coating was invalid in bile salt solution at pH 8.6. This conclusion is questionable because the upper intestine generally has a pH of 5 ~ 6, where bile is excreted (*Notari, 1987*).

1.6.3. *In vitro* Uptake Studies

Since the results of *in vivo* studies are often difficult to interpret or reproduce, several *in vitro* experimental approaches have been adopted in order to determine some of the important factors and mechanisms involved in transport across the gut wall. In particular, three techniques have been used, namely diffusion cells, everted intestinal sacs of the rat and *in situ* perfusion techniques.

The diffusion cell method has been widely used in transdermal studies (*Cooper *and Patel, 1990*) but *Chiang (1986)* introduced this technique to study liposome transport across intestinal membranes. Using this method, a piece of the intestinal segment is placed between a donor (mucosal) and a receiver (serosal) cell. Preparations of test components were applied to the donor compartment and samples were withdrawn periodically from either the donor or the receiver compartment for analysis. The advantage of this technique is that it readily allows sampling from both donor and receiver compartments throughout the experimental period. The major disadvantage is the lack of intact mesentery of the intestinal membrane and, hence, transport is measured only across the epithelium, muscular and connective tissues.

A more physiological model known as modified *Doluisio technique* (*Doluisio et al., 1969; Park et al., 1984*) was used by *Schwinke et al. (1984)* to study liposome intestinal uptake. A section of the rat small intestine was cannulated with two L-shaped cannulae and syringes were attached to each end of the cannulae separately. The sample preparation was then introduced into the intestinal lumen, aliquots were withdrawn and analyzed periodically.

The isolated everted sac technique was developed by *Wilson and Wiseman (1954)* and modified by *Bridges et al. (1978)* to evaluate the effect of uptake of liposome-entrapped macromolecules. The most extensively studied compound has been insulin. A review by *Woodley (1985)* reported that liposome-entrapment protected the insulin from degradation both within the lumen of the sac and during passage across the enterocytes; 95% of the insulin recovered inside the sac after 1 hr incubation was still within liposomes. In contrast, no free drug was able to cross into the serosal compartment. The conclusion was the rat small intestine does have the capability to translocate liposomes from the mucosal to the serosal surface. In addition to insulin, other liposomal compounds have also been found to undergo increased uptake by the intestinal sac, *e.g.* ^{125}I-PVP (*Bridges et al., 1978*), and horseradish peroxidase (*Rowland and Woodley, 1981*). Furthermore, *Rowland and Woodley (1981)* found that the uptake of liposome-entrapped ^{125}I-PVP could be saturated at high concentration and they suggested a mechanism of absorptive endocytosis. In contrast, *Weiner and Chiang (1988)* studied the penetration of liposome entrapped PEG-4000 and glucose and found that free glucose could penetrate into the everted sacs, whereas only small amount of entrapped PEG-4000 and glucose could enter the everted sacs. This apparent contradiction of results has not yet been resolved.

1.6.4. *In vivo* Absorption Studies

The ultimate test of the effectiveness of orally-administered liposomes must be in the living animal, *i.e. in vivo* studies. *Patel and Ryman (1976)* first used liposome-entrapped insulin for oral administration to diabetic rats and found a reduction in the blood glucose levels. This work has since been confirmed and extended by *Dapergolas and Gregoriadis (1977)* and *Hashimoto and Kawada (1979)*. However, no reduction in blood glucose concentration was observed in normal rats after administering liposome-entrapped insulin (*Kowada et al., 1981*). In another study, similar results were obtained after liposomal administration to healthy male volunteers and normal dogs where increased plasma immunoreactive insulin lowered blood glucose in a manner similar to that observed in diabetic dogs (*Patel et al., 1982*). Also, the reduction of glucose levels appeared to dose-independent (*Patel et al., 1978*). However, there are uncertainties regarding the absorption of liposome-entrapped insulin because of very low absorption (about 1%) (*Patel et al., 1978*). Stability studies of liposomes in the GIT revealed that only very small amounts of liposomes were able to survive and reach absorption sites. Therefore, the amount of absorbed contents was very small, even if liposomes did facilitate absorption.

In addition to insulin, the oral absorption of liposome-encapsulated factor VIII was also observed to increase (*Hemker et al., 1980*). *Ryman et al. (1978)* studied the liposome absorption of some nonabsorbable substances, such as inulin, PEG, and gentamicin, but found that less than 1% of administered compounds were absorbed into the blood stream.

In order find a solution to the problem of whether liposomes facilitate drug absorption from the GIT, a stable liposome form must first be prepared. Polymer-coated liposomes with increased stability in the GIT is an important objective in achieving this.

1.7. Aims

Thus far polymer-coated liposomes have not been thoroughly characterized. Hence, the overall aims of this project were:

1. To search for the possible polymers which can be used to coat liposomes as well as feasible methods.
2. To characterize the resulting polymer-coated liposomes, especially the ways of overcoming the liposome instability and leakage of their entrapped drug.
3. To explore the potential usage of improved liposomes for oral administration.

1.8. Hypotheses

The hypotheses of this project were as follows:

1. Liposomes are able to be coated on their surfaces by polymers.
2. The encapsulation efficiency of drugs is not significantly influenced by the polymer coating on the surfaces of liposomes.
3. The physical stability of liposomes in sodium cholate solutions is increased by polymer coating the surfaces of liposomes with polymers.
4. Drug leakage from liposomes can be reduced by the presence of polymers on their surfaces of liposomes.

1.9. Objectives

Based on the above hypothesis, the following objectives were identified to achieve our aims:

1. Develop the coating techniques on the surfaces of liposomes for the three selected polymers, poly-4-vinylpyridine, carboxymethyl chitin, and acacia-gelatin.
2. Assess the encapsulation efficiency of ASA in polymer-coated liposomes.
3. Study the particle sizes of polymer-coated liposomes.
4. Evaluate the physical stability of polymer-coated liposomes in sodium cholate solutions.
5. Determine the relative drug release characteristics from polymer-coated liposomes.

1.10. References

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CHAPTER 2

POLY(4-VINYLPYRIDINE)-COATED LIPOSOMES. PREPARATION, STABILITY, AND RELEASE OF ACETYLSALICYLIC ACID[1]

2.1. Introduction

A serious limitation to the use of liposomes has been their relatively poor stabilities during storage and following administration (*Woodley, 1985*). Attempts to overcome this problem have led to the development of polymerized liposomes (*Albrecht et al., 1986; Regen et al., 1980 & 1981*), or polymer-coated liposomes (*Regen et al., 1984*). In the former case, a decreasing interest in further development stems from difficulties associated with the complexity of the process, toxicities of the reagents used, and limited control over the permeabilities obtained. On the other hand, functionality of polymer-coated liposomes has been repeatedly demonstrated (*Aliev et al., 1984; Kato et al., 1985; Seki and Tirrell, 1984; Takada et al., 1984*). It would be particularly advantageous to have a liposome preparation that was stable in the GIT (*Woodley, 1985*) and liposomes coated with polymers have the potential of achieving this objective.

Several mechanisms involved in the coating of liposomes include, adsorption of polymers at liposome surfaces with attachment by hydrophilic or ionic forces (*Kato et*

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al., 1985; Seki and Tirrell, 1984), fixation of polymers by hydrophobic anchor groups (*Takada et al., 1984*), binding of polymerizable, water-soluble monomers to liposome surfaces by ionic interactions (*Aliev et al., 1984; Ringsdorf and Schlarb, 1986*), or cleavage of a spacer group attached to a polymer backbone after its incorporation into liposomes (*Regen et al., 1984*).

Aliev et al. (1984) reported that after salt formation of 4-vinypyridine (4-VP) with negatively-charged dicetylphosphate liposomes, autopolymerization of the 4-VP occurred to form poly(4-vinylpyridine) (poly(4-VP)) which coated the liposomes. The physical stability of the coated liposomes and their application as a drug delivery system were not addressed. Thus, a study was undertaken to determine the optimum conditions of coating liposomes with this type of polymer, to find out what effect this would have on the stability of the liposomes in certain destabilizing vehicles, and to measure the encapsulation efficiency of a solute and its release kinetics from this type of polymer-coated liposome.

2.2. Materials and Methods

2.2.1. Materials

Acetylsalicylic acid (ASA), sodium cholate, 4-vinylpyridine (4-VP), and deuterium oxide (99.8%) were obtained from Aldrich Chemical Co. L-α-dimyristoylphosphatidylcholine (DMPC, 98%) and dicetylphosphate (DCP) from Sigma Chemical Co. were used as received. Acetonitrile was received from BDH Inc. All other materials were at least reagent grade.

2.2.2. Preparation of Poly(4-VP)-Coated Liposomes

Liposomes of DMPC/DCP were prepared by the hydration method originally described by *Bangham et al. (1965)*. Briefly, 36 μmol of DMPC and 36 μmol of DCP were dissolved in 10 ml of chloroform. The organic solvent was removed by rotary evaporation under reduced pressure at 50°C. The formed lipid film flushed with nitrogen was put into a vacuum oven at 30°C for 8 hr for further drying, then hydrated with 5 ml of pH 4.0 acetate buffer (171.2 mM acetic acid, 35 mM sodium acetate) at 63°C and vortex-mixed until fully hydrated. After cooling to room temperature, 42.5 μmol of 4-VP was mixed with the liposomes and polymerization of 4-VP was carried out at room temperature for 2 hr. Excess 4-VP was removed from the supernatant after centrifugation at 135,000 ×g for 20 min (Beckman Ultracentrifuge, Model L8-55) and the resulting pellet was washed twice with pH 4.0 buffer. When ASA (30 mM) was included it was incorporated together with the phospholipids in the organic solvent prior to the hydration stage.

2.2.3. Poly(4-VP) Coating Efficiency

The coating efficiency of poly(4-VP)-coated liposomes was determined by dissolving the sediment of a centrifuged sample in chloroform:isopropanol (1:4 v/v) and analyzing for poly(4-VP) content by HPLC (solvent delivery system, Waters Model 501; variable wavelength detector, Waters Lambda-Max, Model 481 set at 261 nm; Rheodyne injector, Spectra Physics Model 7125; 20 μl injection loop; C_{18} reverse phase chromatography column, Waters Novapak, 15 cm, 5 μm; integrator-plotter, Waters Model 740). The mobile phase was 70% pH 5.6 buffer (0.157 M sodium acetate, 0.015 M acetic acid), and 30% acetonitrile delivered at a flow rate of 1.5 ml/min. The coating efficiency of poly(4-VP) was expressed as the weight ratio of poly(4-VP):lipid (percentage), using poly(4-VP) extracted from poly(4-VP)-coated liposomes (*Aliev et al., 1984*) and confirmed via ^{1}H-NMR (Bruker AM-300 MHz Spectrophotometer).

2.2.4. Electron Microscopy

A drop of the liposome preparation was applied to a formvar film-coated copper grid and the excess was removed with a filter paper. Phosphotungstic acid solution (1%, pH 7.0 adjusted with KOH solution) was dropped on the copper grid. After 10 seconds of staining, it was removed with a filter paper. Consequently, the specimens were examined with a Hitachi H-7000 electron microscope at 75 KV.

2.2.5. Liposome Size and Poly(4-VP) Coating Thickness

A liposome preparation was diluted with pH 4.0 acetate buffer (40 μl of liposomes to 3 ml of buffer) then sized by dynamic light scattering (Brookhaven Instruments Particle Sizer, Model BI-90) according to the following regimen: 2500 cycles/run, 3 to 9 runs for each sample at a count rate of about 18 kcps. Triplicate determinations were made of all samples. The thickness of the poly(4-VP) coating was estimated from the diameter differences between poly(4-VP)-coated and uncoated (control) liposomes.

2.2.6. Liposome Stability

The relative stability of poly(4-VP)-coated liposomes was assessed by employing a turbidimetric method (*Regen et al., 1980; Rogers et al., 1990*). Liposomes were mixed with sodium cholate or isopropanol solutions then diluted to 10 ml with pH 7.0 acetate buffer solution. Turbidities were measured spectrophotometrically at 400 nm (Beckman spectrophotometer, Model 25) the intensities of which were indicative of the degree of dissolution of the liposomes relative to the control.

2.2.7. ASA Encapsulation Efficiency

Liposome-encapsulated ASA was separated from free ASA by centrifugation at 135,000 ×g for 20 min. The pellet containing entrapped ASA was dissolved in chloroform:isopropanol (1:4 v/v) then quantitative analysis of ASA was carried out by HPLC at 280 nm using a mobile phase of 20% acetonitrile aqueous solution containing 0.1% phosphoric acid at pH 2.7 delivered at a flow rate of 1.5 ml/min. The encapsulation efficiency was calculated as percentage of ASA encapsulated in the liposomes over the total ASA added.

2.2.8. Release Rate of ASA

A batch (5 ml) of poly(4-VP)-coated liposomes containing ASA was centrifuged at 135,000 ×g for 20 min. After removing the supernatant, the pellet was washed with pH 7.0 buffer, resuspended in 10 ml pH 7.0 phosphate buffer and incubated at 37°C. At 20 min intervals, 1 ml samples were withdrawn and centrifuged immediately at 135,000 ×g for 10 min. The supernatant was analyzed for both ASA and its degradation product, SA, by HPLC and the rate of release determined as follows,

$$\text{ASA release (\%)} = (C-C_o)/(C_t-C_o) \times 100$$

where C_o is the initial ASA concentration in the supernatant before incubation, C_t the total ASA and SA concentrations in the liposome suspension, and C the ASA and SA concentrations in the sample supernatant after time t.

2.3. Results and Discussion

2.3.1. Polymerization of 4-VP on the Surfaces of Liposomes

The mechanisms involved in the poly(4-VP) coating of DMPC/DCP liposomes may be depicted as shown in Fig. 2.1. Positively-charged 4-VP monomers are electrostatically attracted to the centers of negative charges of DCP at the liposome surfaces forming the salt, $[\text{4-VP}]^+[\text{DCP}]^-$. Subsequently, within a few minutes, autopolymerization occurred to form poly(4-VP)-coated liposomes. The UV absorption profiles of 4-VP and poly(4-VP) differ, hence, the polymerization of 4-VP can be followed by scanning UV. Neither DMPC nor DCP in the solution caused interference in this determination. Comparative spectra of 4-VP and poly(4-VP) arising from autopolymerization at the liposome surfaces are shown in Fig. 2.2. In acidic iso-propanol solution, the spectrum of 4-VP exhibited a major peak at 261 nm and a minor peak at 206 nm, whereas a major peak at 209 nm and a minor peak at 262 nm were observed with poly(4-VP) after 40 min following the addition of 4-VP (8.5 mM) to the DMPC/DCP liposomes. These results concurred with those reported by *Aliev et al.*

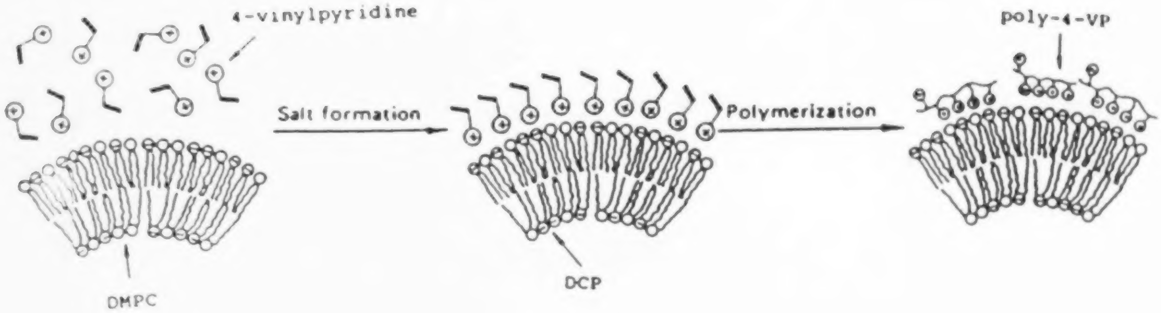
4-vinylpyridine
Salt formation
Polymerization
poly-4-VP
DMPC
DCP

Fig. 2.1. Mechanisms of 4-VP autopolymerization on the surface of DMPC:DCP liposomes.

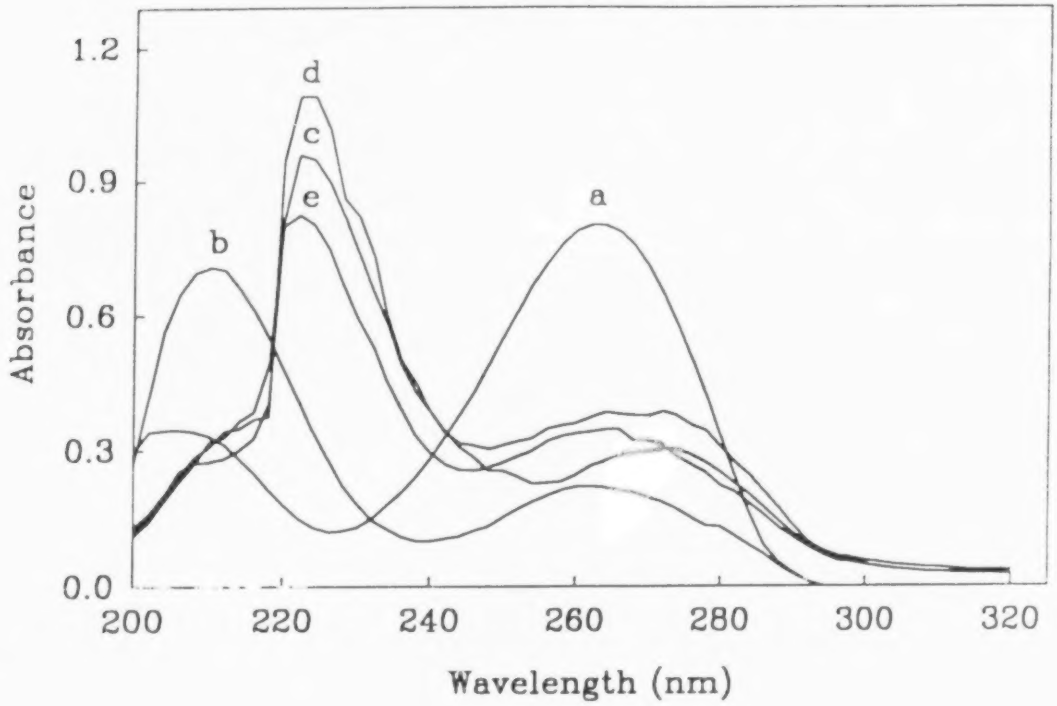

Fig. 2.2. Ultraviolet spectra of acidic isopropanol solutions of: a. 4-VP and DMPC:DCP liposomes 40 min after being coated with, b. 8.5 mM 4-VP; c. 20 mM 4-VP; d. 40 mM 4-VP; e. 60 mM 4-VP.

(1984), indicating that polymerization of 4-VP had taken place. The rate of autopolymerization of 4-VP at the liposome surfaces also depended on the DCP content. At a DCP content of 33 mole% or above, polymerization was essentially complete within 40 min, whereas the process was not completed until after 5 days when only 20 mole% of DCP had been incorporated. This indicates a dependency of the coating and polymerization processes on the density of negative charges at the liposomal surfaces.

A negative stain electron micrograph of poly(4-VP)-coated liposomes can be distinguished clearly from one of uncoated liposomes (Fig. 2.3). In contrast to control liposomes, the polymer-coated vesicles show filament structures on the surface of liposomes. Positive polymer is able to bind the negative phosphotungstic acid as a counterion and therefore becomes negatively stained.

2.3.2. Poly(4-VP) Coating Efficiency

The coating efficiency of liposomes by poly(4-VP) as a function of the DCP content at different initial 4-VP concentrations is shown in Fig. 2.4. At 8.5 mM 4-VP, the coating efficiency was low and the coating efficiencies for the different compositions were not statistically different (F-test, p=0.05), even though on apparent peak occurred at above 33 mole% DCP in the liposomes. However, at 20 mM or 40 mM 4-VP, the coating efficiencies were considerably greater than at 8.5 mM but at these tests their differences were not significant from each other (F-test, p=0.05). At 60 mM 4-VP the coating efficiency was greatest at 20 mole% DCP in the liposomes

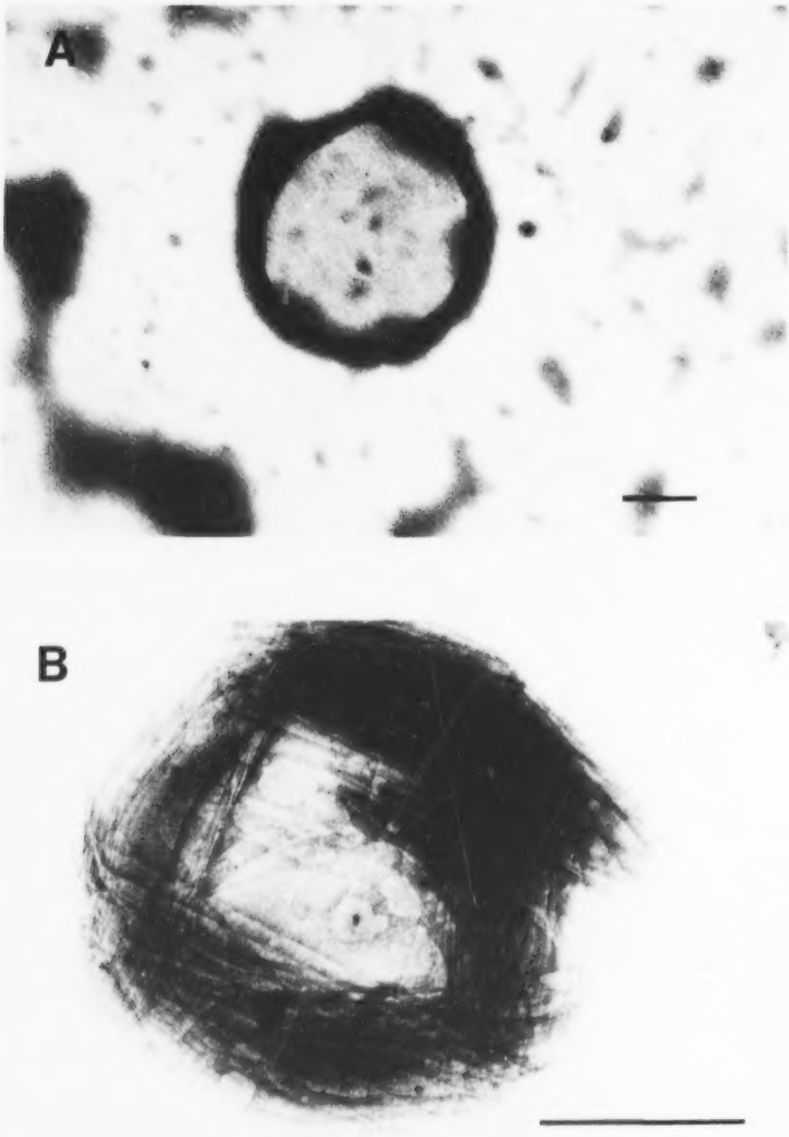

Fig. 2.3. Transmission electron micrographs of: A) DMPC/DCP liposomes and, B) poly(4-VP)-coated liposomes negatively stained with phosphotungstic acid. Bar length = 1 μm.

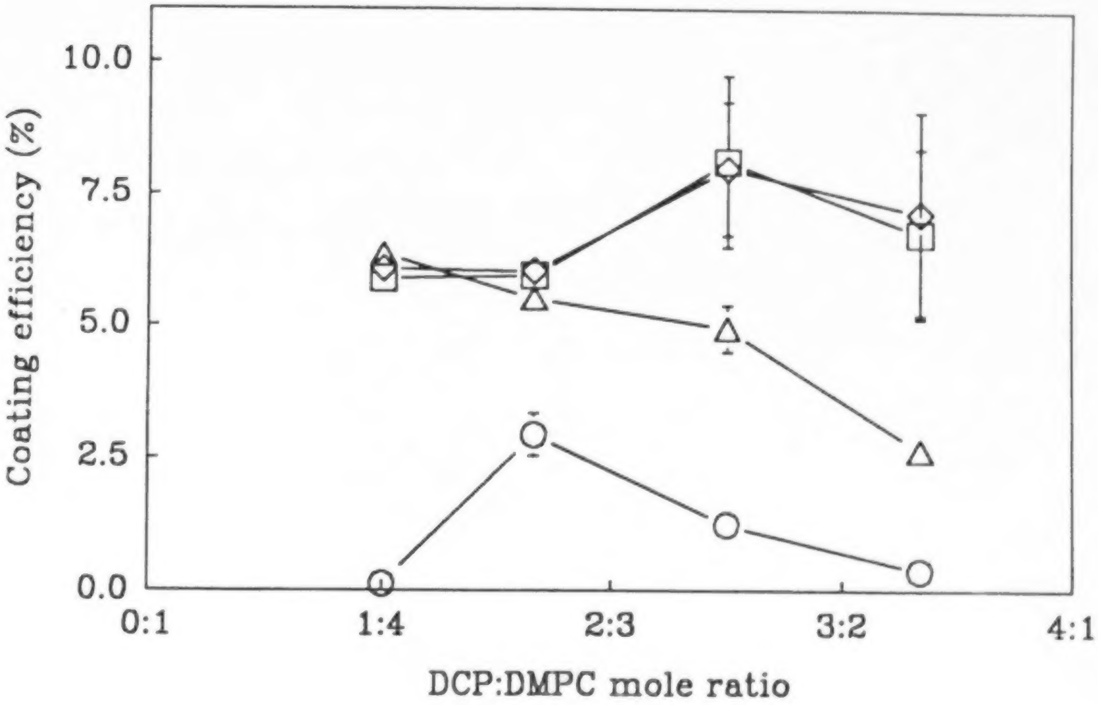

Fig. 2.4. Poly(4-VP) coating efficiency (polymer:lipid weight ratio, %) of DMPC/DCP liposomes as a function of DMPC content in pH 4.0 sodium acetate buffer at 22°C with a total lipid concentration of 14.4 mM and initial 4-VP concentration of, ○ , 8.5 mM; □ , 20 mM; ◇ , 40 mM; and △ , 60 mM. ± SD (n=3).

but then decreased sharply as the DCP content of liposomes was increased. A possible explanation of this behavior is interference in the autopolymerization process due to excess salt formation and disruption of the liposomal bilayer structure. The coating efficiency of DMPC/DCP (1:1 mole ratio) liposomes by poly(4-VP) was also a function of the pH of the medium (Fig. 2.5). DCP has a pK_a of 4.5 (*Maitain et al., 1990*) and 4-VP has a pK_a of about 6 according to spectrophotometric titration (*Wu et al., 1971*) and 5.72 according to calculation (Appendix). At each of the initial 4-VP concentrations the coating efficiency reached a maximum at a pH of 4.0, because at this pH the dissociation of DCP and 4-VP becomes optimal and the reacting ions attain the strongest electrostatic interaction.

The poly(4-VP) coating efficiency as a function of the initial 4-VP concentration at two different polymerization times is described in Fig. 2.6. At initial 4-VP concentrations up to 37 mM and a polymerization time of 20 min, liposome coating by poly(4-VP) was linear, *i.e.* adhering to mass action principles. At 37 mM, the coating efficiency reached a maximum indicating saturation of the available binding sites at the bilayer surfaces by 4-VP. However, after 140 min of polymerization time, the coating efficiency peaked at an initial 4-VP concentration of 20 mM and decreased at an initial 4-VP concentration of 60 mM (F-test, p=0.05). Thus, polymerization appears to limit the number of binding sites available for salt formation by excess 4-VP. Hence, shorter polymerization times are more efficient than longer times in achieving increased levels of 4-VP attachment to the liposomes. In this regard, the liposome size is shown to increase as a function of the initial 4-VP concentration in Table 2.1. The average

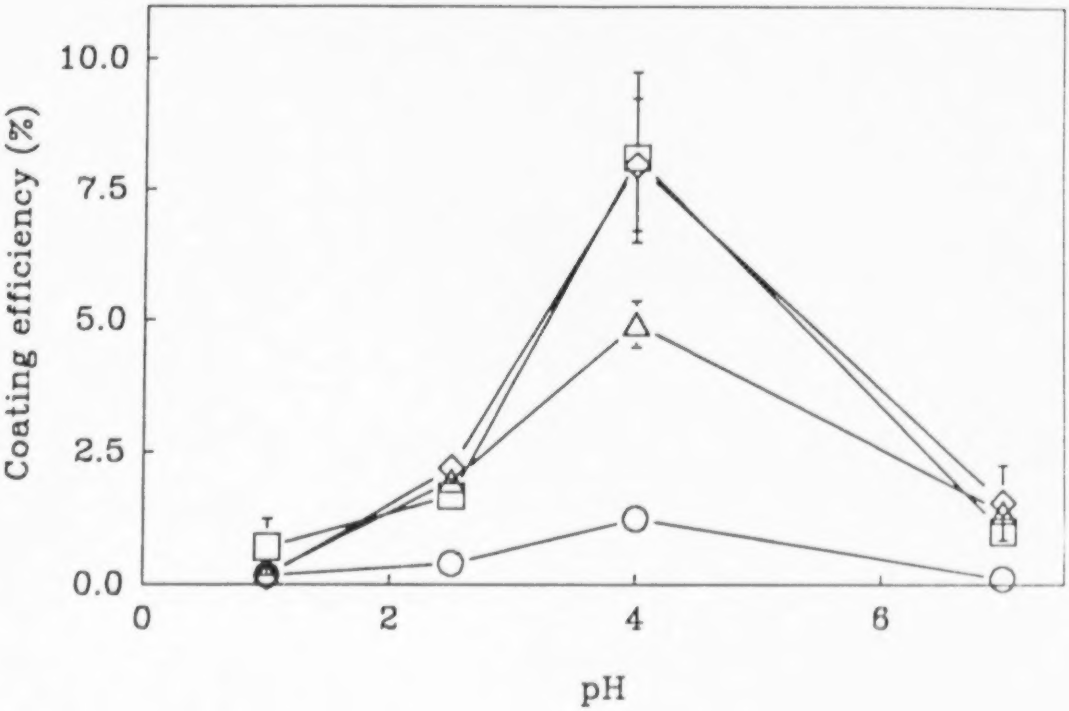

Fig. 2.5. Poly(4-VP) coating efficiency (polymer:lipid weight ratio, %) of DMPC/DCP (1:1 mole ratio) liposomes as a function of pH at 22°C with a total lipid concentration of 14.4 mM and initial 4-VP concentration of, ○ , 8.5 mM; □ , 20 mM; ◇ , 40 mM; and △ , 60 mM. ± SD (n=3).

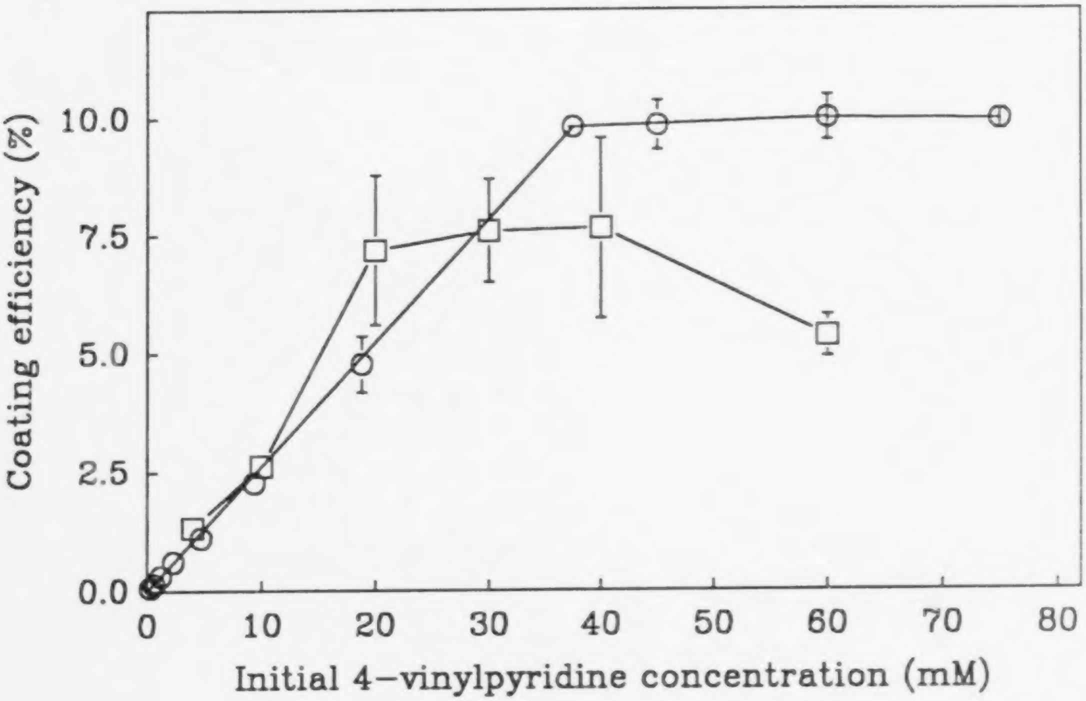

Fig. 2.6. Poly(4-VP) coating efficiency (polymer:lipid weight ratio, %) of DMPC:DCP (1:1 mole ratio) liposomes as a function of initial 4-VP concentration at a total lipid concentration of 14.4 mM in pH 4.0 sodium acetate buffer at 22°C. ○ , 40 min and, □ , 140 min of polymerization. ± SD (n=3).

Table 2.1. Mean diameter, and size range of poly(4-VP)-coated liposomes by dynamic light scattering[a].

Initial 4-VP Conc. (mM)	Average diameter[b] (μm)	Diameter range (μm, p=0.1)
0	1.61 (0.18)	0.59 ~ 2.97
8.5	1.94 (0.78)	0.62 ~ 3.73
20	2.38 (0.08)	0.69 ~ 4.69

[a] Total lipid = 14.4 mM (DMPC/DCP 1:1 mole ratio); n=3.

[b] SD in brackets.

size of uncoated liposomes was 1.6 μm, ranging from 0.59 to 2.97 μm and there was a significant increase in the average size of liposomes after poly(4-VP) coating (F-test, $p=0.05$), with 20 mM 4-VP.

2.3.3. ASA Encapsulation Efficiency

The encapsulation efficiency of ASA in liposomes at pH 4.0 as a function of DMPC:DCP mole ratio is shown in Fig. 2.7. The ASA encapsulation increased as a function of the DMPC:DCP mole ratio reaching a maximum of 9% at a DMPC:DCP 1:1 mole ratio. Above a DMPC:DCP 1:1 mole ratio, the encapsulation of ASA decreased abruptly to about 5% then remained constant. Since 76% of ASA is ionized at this pH, a fraction of its uptake must be considered as being in the aqueous compartments. The volumes of the aqueous compartments are reportedly greater in negatively charged than in neutral liposomes (*Chapman, 1984*). Hence, it is probable that above a 1:1 mole ratio DMPC:DCP a reduction in the volume of the aqueous compartments of the liposomes occurs, thereby reducing the entrapment of ionized ASA. The partial specific volume of DMPC:DCP liposomes was, in fact, shown to decrease above a 2:1 mole ratio (*Rogers and Cheuk, 1990*). Pure DCP was used to make liposomes which obtained an encapsulation efficiency of 0.9%. The formation of liposomes was not confirmed although *Ringsdorf (Aliev et al., 1984)* had formed the liposomes from pure DCP.

Table 2.2. shows the ASA encapsulation efficiency results in poly(4-VP)-coated liposomes as a function of both initial 4-VP concentration at pH 4.0 and polymerization

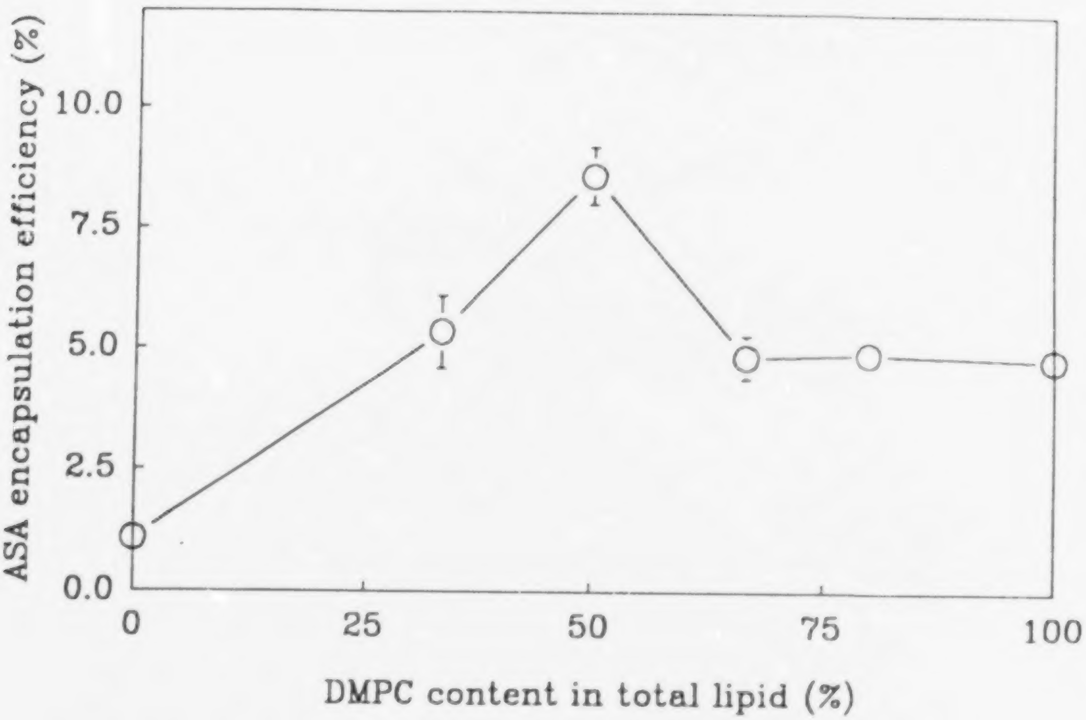

Fig. 2.7. The encapsulation efficiency of ASA in liposomes as a function of DMPC:DCP mole ratio in pH 4.0 sodium acetate buffer. Total lipid concentration was 14.4 mM and initial ASA concentration was 30 mM.

Table 2.2. Percent ASA encapsulation efficiency in poly(4-VP)-coated liposomes as a function of initial 4-VP concentration and 4-VP polymerization time[a].

Initial 4-VP conc. (mM)	Time of 4-VP polymerization[b] (min)			
	20	80	140	500
8.5	7.8 (0.3)	7.8 (0.6)	8.1 (0.6)	7.9 (1.5)
20	7.7 (1.7)	8.1 (1.5)	6.7 (0.8)	7.5 (1.4)
40	5.7 (0.1)	5.4 (0.2)	4.6 (0.4)	0.9 (0.3)
60	3.7 (0.5)	2.7 (0.6)	1.2 (0.2)	0.9 (0.3)

[a] Total lipid = 14.4 mM (DMPC/DCP 1:1 mole ratio); n = 3; encapsulation efficiency of uncoated liposomes was 7.7 (0.9).

[b] SD in brackets.

time. It can be seen that the encapsulation of ASA remained essentially unchanged at initial 4-VP concentrations ranging from 8.5 to 20 mM and a polymerization time up to 500 min. However, raising the initial 4-VP concentration to 40 mM caused leakage of ASA and approximately 90% had been released after 500 min, possibly due to the formation of 1,2-poly(4-VP) which destroyed the liposomes. At 60 mM 4-VP it is obvious that the liposomes were sufficiently perturbed to cause rapid and extensive leakage of ASA.

2.3.4. Physical Stability

The physical stabilities of uncoated and poly(4-VP)-coated liposomes in sodium cholate solutions or diluted isopropanol solutions are described in Figures 2.8 and 2.9, respectively, as a function of the initial 4-VP concentration at pH 7.0. At an initial 4-VP concentration of 8.5 mM, higher turbidities were recorded for poly(4-VP)-coated liposomes than uncoated liposomes in either sodium cholate or diluted isopropanol solutions indicating that the polymer coating imparted greater resistance of the liposomes to dissolution. The polymer-coated liposomes were considered to be significantly stable in 2 mM sodium cholate (F-test, $p=0.05$) or 4% isopropanol solutions whereas a 20~30% decrease in stability occurred with uncoated liposomes. In contrast, the stability of poly(4-VP)-coated liposomes, coated from an initial 4-VP concentration of 20 mM, was less than uncoated liposomes at pH 7.0 in either sodium cholate or hydroalcoholic solutions even though this represented maximum coating efficiency at pH 4.0. The autopolymeriza-tion of 4-VP has previously been explained

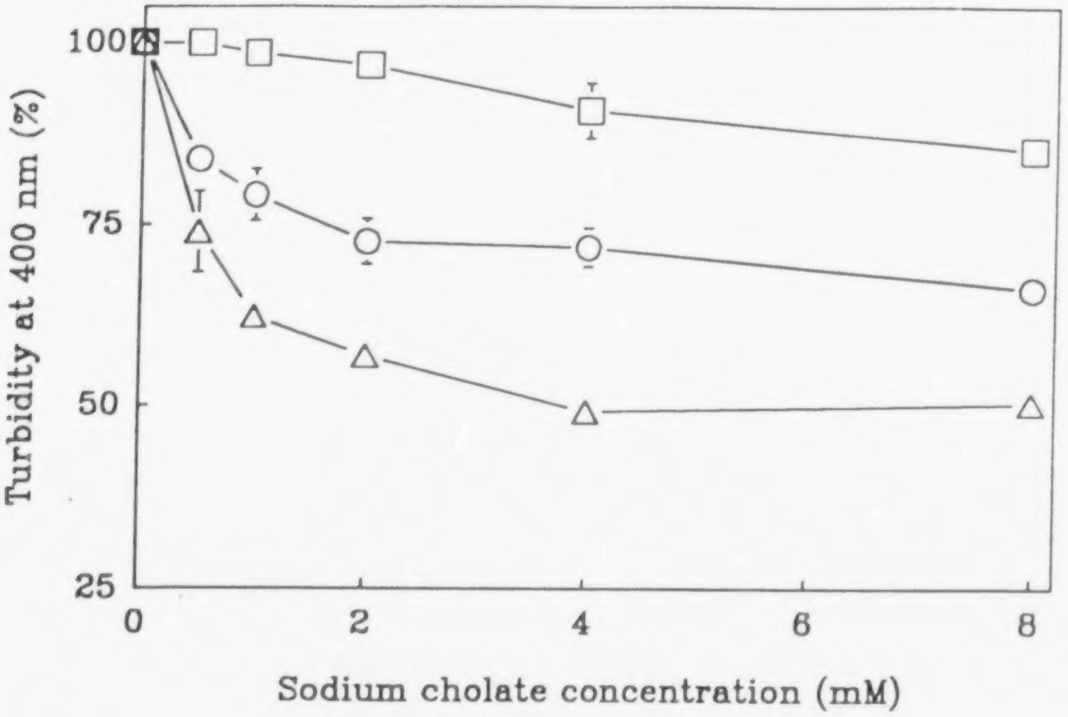

Fig. 2.8. Comparison of the relative stability of uncoated, ○ , and poly(4-VP)-coated liposomes with initial 4-VP concentration of, □ , 8.5 mM or, ▵ , 20 mM against sodium cholate solutions in pH 7.0 phosphate buffer at 22°C. ± SD (n=6).

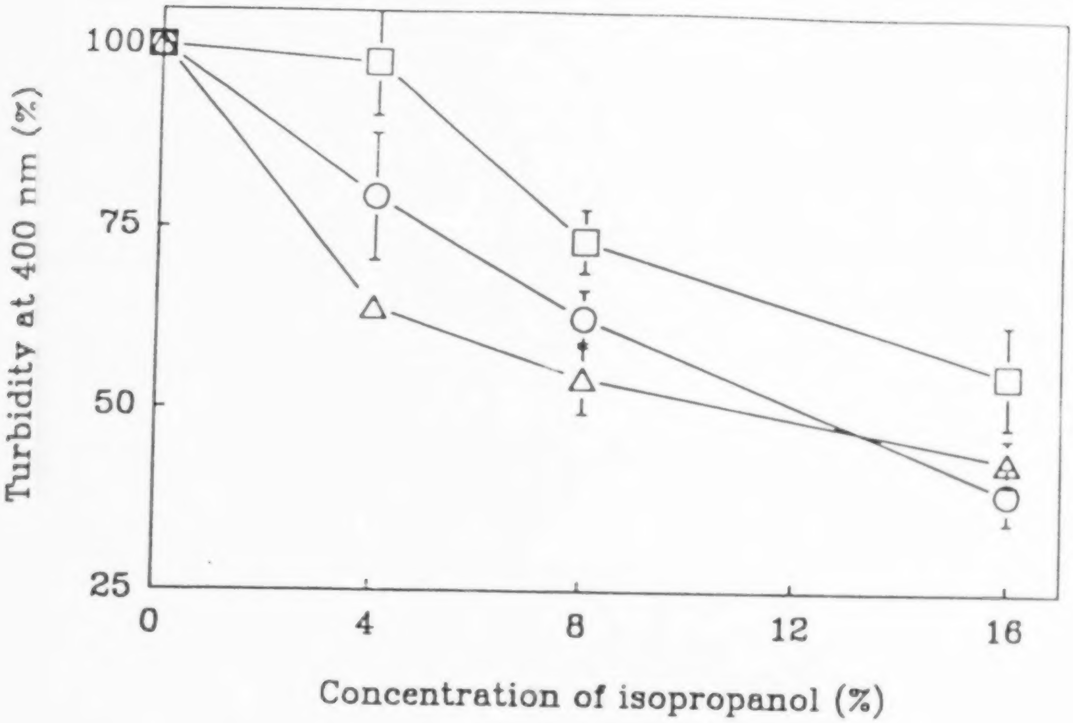

Fig. 2.9. Comparison of the relative stability of uncoated, ○ , and poly(4-VP)-coated liposomes with initial 4-VP concentration of, □ , 8.5 mM or, △ , 20 mM against aqueous isopropanol solutions in pH 7.0 phosphate buffer at 22°C. ± SD (n=6).

to yield either 1,6-poly(4-VP) or 1,2-poly(4-VP) at low or high initial 4-VP concentrations, respectively (*Aliev et al., 1984*). Figure 2.2c, 2.2d, and 2.2e are the scanning UV curves obtained from poly(4-VP) derived from autopolymerized 4-VP at liposome surfaces at 20 mM, 40 mM, and 60 mM initial concentrations, respectively. The major peak at 220 nm in these instances corresponds to 1,2-poly(4-VP) (NMR spectrum not shown) whereas Figure 2b corresponds to the 1,6-poly(4-VP), as confirmed by NMR spectal analysis (Fig. 2.10). The spectrum shows two groups of peaks caused by the aromatic ring protons (~7.9 and 8.8 ppm) and two groups of peaks caused by the aliphatic methylene protons (3.7 and 5.0 ppm). Proton integration shows that four groups have equal number of protons which corresponds to two protons in each group of peaks. The peak at 3.1 ppm corresponds to an impurity. The terminal methyl protons of the polymer give too weak a signal to be seen. Thus, the decreased liposome stability and increased leakage of ASA from poly(4-VP)-coated liposomes occurred as a result of formation of 1,2-poly(4-VP) which participates with the sodium cholate or isopropanol to solubilize the liposomes. At 8.5 mM initial 4-VP, liposomes are coated with a stable 1,6-poly(4-VP) film which reduces leakage of ASA (Table 2.2). In any case, dilute sodium cholate or isopropanol solutions were equally effective for determining the relative stabilities of these liposome preparations.

2.3.5. ASA Release

The leakage of ASA from poly(4-VP)-coated liposomes at an initial 4-VP concentration of 8.5 mM was decreased compared with uncoated liposomes (Fig. 2.11).

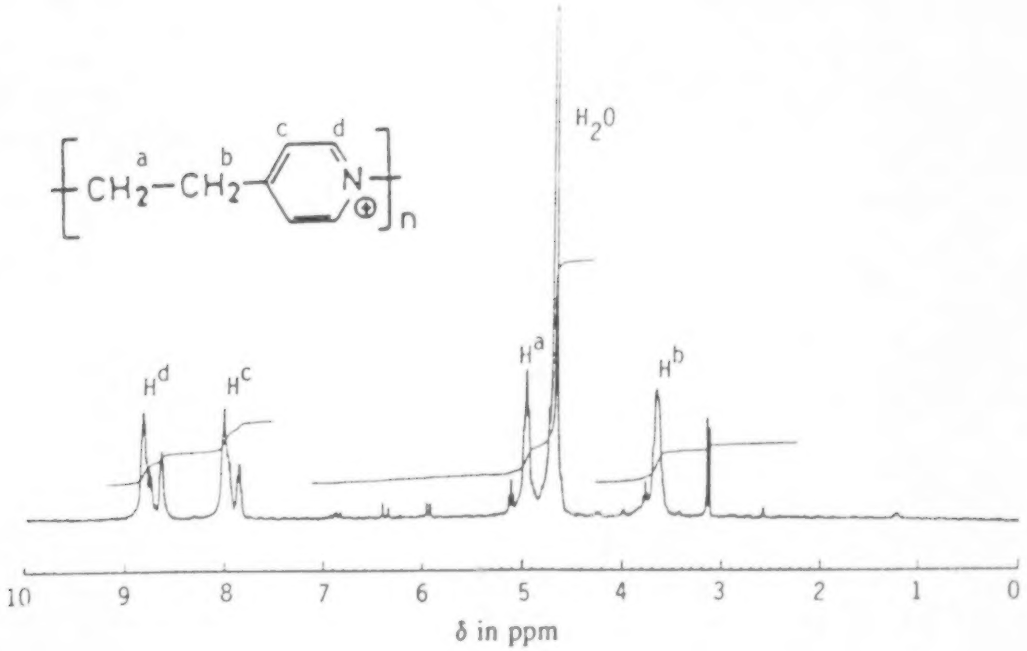

Fig. 2.10. 300 MHz ^{1}H-NMR spectrum of poly(4-VP) isolated from DMCP/DCP liposomes which were coated with an initial 4-VP concentration of 8.5 mM.

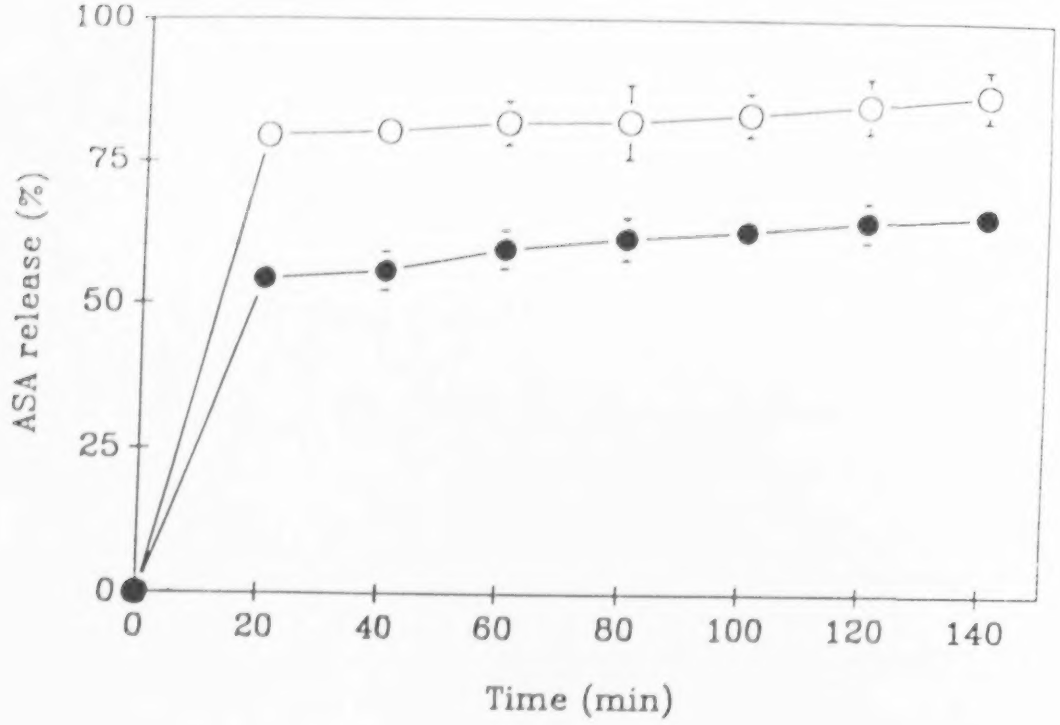

Fig. 2.11. ASA release from poly(4-VP)-coated DMPC/DCP liposomes in pH 7.0 phosphate buffer at 37°C. ○ , uncoated liposomes; ● , poly(4-VP)-coated liposomes with an initial 4-VP concentration of 8.5 mM. ± SD (n=3).

In the first 20 min, more than 85% of the encapsulated ASA was released from uncoated liposomes, compared to only 63% from poly(4-VP)-coated liposomes. The initial fast release could possibly have been due to slight osmotic effects related to the ASA in the liposomes but most likely it was due to the change in temperature from R.T. to 37°C. The leakage of the remaining ASA from liposomes was so slow that there was no significant amount of ASA released after 2 hours for either the polymer-coated or the uncoated liposomes (F-test, $p=0.05$).

2.4. Summary

The results of this study demonstrate a clear effect of the coating of DMPC/DCP liposomes by poly(4-VP) on liposome stability and the release of ASA. The effects produced were somewhat dependent on the initial 4-VP concentration used. Poly(4-VP)-coated liposomes coated with an optimum initial 4-VP concentration of 8.5 mM appeared to have a greater stability and slower release of ASA than uncoated liposomes. Overall, these results offer some promise of having a more stable liposome which has greater retention of solute that may be beneficial for the improved bioavailability of certain types of drugs. The rapid and convenient means of placing a polymer coating on liposomes using this technique is an advantage although the *in vivo* toxicity of poly(4-VP) is as yet unknown.

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CHAPTER 3

CM-CHITIN-COATED LIPOSOMES

CHAPTER 3

Part A

QUANTITATIVE DETERMINATION OF CM-CHITIN IN POLYMER-COATED LIPOSOMES[2]

3A.1. Introduction

Carboxymethyl chitin (CM-chitin), a derivative of a poly-(1-4)-linked N-acetyl-D-glucosamine, has been used to stabilize egg yolk phosphatidylcholine vesicles (*Izawa et al., 1986*), especially in liposome-type artificial red blood cells (*Kato et al., 1984*). Used in this manner, CM-chitin is electrostatically bound to the surface of the liposomes thereby strengthening the liposomes and providing increased stability to the vesicles (*Kato et al., 1983 & 1987*). No quantitative direct determination of CM-chitin in the presence of liposomes has been reported. *Clark et al. (1977)* employed a method of polysaccharide determination involving the analysis of the mixture of monosaccharides that is released from the polymer by hydrolytic or enzymatic digestion. However, this method is inconvenient and does not particularly lend itself to quantitative determination of CM-chitin, especially in liposome systems.

The aim of this investigation was to develop a convenient, quantitative determination of CM-chitin in coated liposome systems using a turbidimetric method.

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[2] A version of this part has been published in *J. Microencapsulation*, 8, 153-160, 1991.

3A.2. Materials and Methods

3A.2.1. Materials

CM-chitin was obtained from Nanyo Kasei Co., Ltd.,Tokyo, Japan. Dimyristoylphosphatidylcholine (99%, DMPC), dipalmitoylphosphatidylcholine (99%, DPPC), distearoylphosphatidylcholine (99%, DSPC), and dicetyl phosphate (DCP) (*Sigma Chemical Co.*) were used as received. Egg phosphatidylcholine (EPC) was extracted from eggs according to the method of *Singleton et al. (1958)* then purified by column chromatography over alumina. Fractions, which yielded a single spot by TLC, were collected and stored at 5°C prior to use. All other chemicals were reagent grade and water was twice-distilled.

3A.2.2. Selection of Solvent System

Several solvent systems, including methanol, ethanol, isopropanol, acetone and their aqueous solutions, were tested for their solvency of phospholipids but their nonsolvent action for CM-chitin by visual inspection and by spectrophotometric means at 400 nm (*Beckman Spectrophotometer, Model 25*).

3A.2.3. Preparation of Standard Solutions

Standard solutions of CM-chitin were prepared by diluting a 1% stock solution in water (20-200 μl) to 5 ml with sodium acetate buffer solution at pH 2.5, 4.0, or 5.6, then further dilution to 10 ml with isopropanol.

3A.2.4. Preparation of Samples

Samples were prepared containing phospholipid and CM-chitin in the same manner as for the standard solutions, except the isopropanol for dilution contained the required amounts of phospholipid.

3A.2.5. Coating Efficiency of DPPC Liposomes by CM-chitin

Liposomes were prepared by the reverse-phase evaporation method (*Szoka et al., 1978*). Briefly, phosphate buffer solution (pH 7.0) was emulsified in dichloromethane containing DPPC, then sonicated (*Model W-375 sonicator, Heat Systems-Ultrasonics, Inc., cup-horn, model 431*) at 10 Khz for 4 min. Subsequently, CM-chitin solution was added to the w/o emulsion and vortex-mixed to form a w/o/w emulsion. The organic solvent was slowly removed under reduced pressure by rotary evaporation resulting in polymer-coated liposomes. The CM-chitin-coated liposomes were then separated from free CM-chitin by gel permeation chromatography (*Sephadex G-25, 1.6 × 70 cm*) using pH 5.6 acetate buffer solution as the eluting solvent. Fractions of the coated liposomes eluted from the column were collected and pooled, allowed to sediment (~12 hr), the supernatant was removed by Pasteur pipette, then the sediment was diluted to 10 ml with buffer solution. Following this treatment, 5 ml were removed and mixed with 5 ml of isopropanol and the turbidty was measured spectrophotometrically at 400 nm. In some experiments pre-formed liposomes prepared by this method were diluted with CM-chitin solution, equilibrated, and treated as before. The coating efficiency of liposomes by CM-chitin was calculated as the ratio of the weight of CM-chitin (mg) determined from the polymer-coated liposomes to the weight of DPPC per ml of liposome suspension (25 mg).

3A.3. Results and Discussion

3A.3.1. Selection of Solvent

Aqueous solutions of methanol, acetone, ethanol, or isopropanol were poor solvents for CM-chitin but had varying solvent effects on liposomal phospholipids. However, among these only aqueous isopropanol solutions were able to dissolve DMPC, DPPC, or EPC and, thus, were chosen as the solvent systems at 1:1 v/v aqueous buffer:isopropanol. The sensitivity of the measurement (*i.e.* degree of turbidity) was a function of the wavelength as depicted in Fig. 3A.1. Thus, the sensitivity of CM-chitin determination decreased as the wavelength of detection increased as predicted according to ***Raleigh's law*** of light scattering (***Wieding et al., 1989***). The wavelength of 400 nm was considered optimum for sensitivity in the visible light range and has also been previously chosen by others for turbidity measurements (***Regen et al., 1980***).

3A.3.2. CM-chitin Turbidity at Different pH

Fig. 3A.2 describes the effect of pH on the sensitivity of measurement at 100 μg/ml CM-chitin and an ionic strength of 0.2. The results gave a maximum turbidity at pH 2.0 ~ 2.5 and 5.6 but a mimimum at pH 3.5 and negligible turbidity at pH 1.0 or above pH 7.0. The disappearance of turbidity at low and high pH values can be

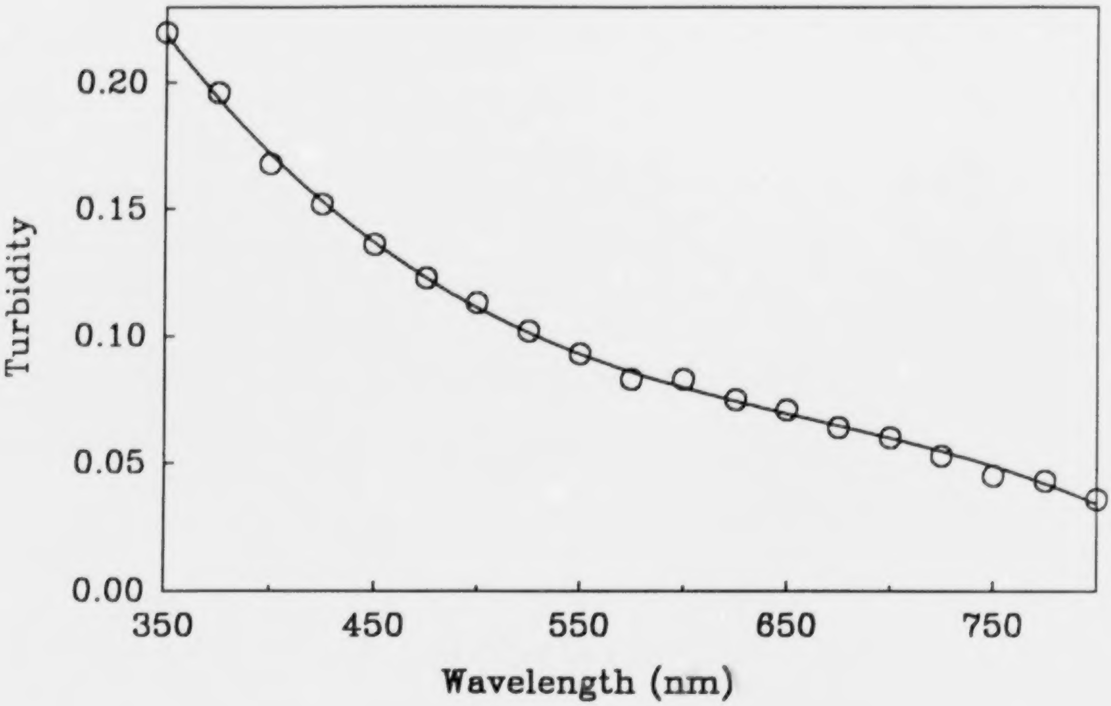

Fig. 3A.1. CM-chitin turbidity as a function of the wavelength of light. The solvent system was 1:1 v/v isopropanol:acetate buffer solution (pH 5.6); the initial CM-chitin concentration was 100 μg/ml; the ionic strength was 0.2.

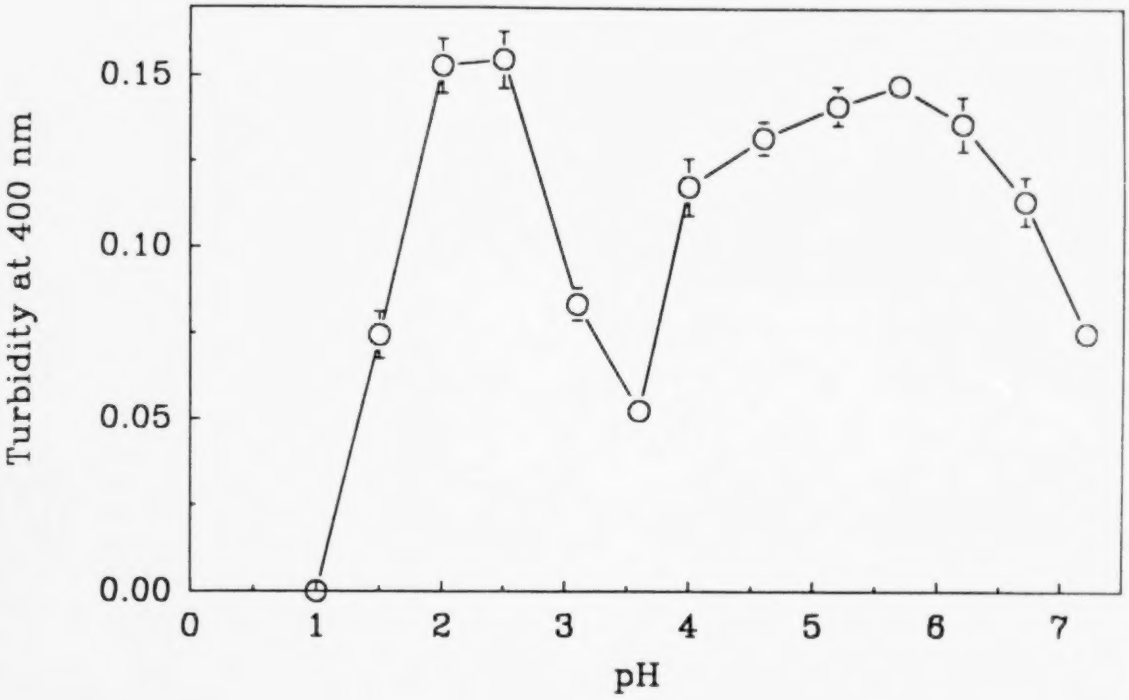

Fig. 3A.2. Dependence of the CM-chitin turbidity on the pH in 1:1 v/v isopropanol:acetate buffer solution. The CM-chitin concentration was 100 μg/ml; the ionic strength was 0.2.

explained by the high degree of ionization of the glucosamine residues or the carboxyl groups of CM-chitin, respectively, giving rise to increased solubility in the aqueous solvent system. On the other hand, it is possible that only partial ionization of the basic and acidic groups at pH 2.0 ~ 2.5 and 5.6 resulted in a minimum in solubility of CM-chitin in the solvent system and the corresponding maxima in turbidity, respectively. The reason for decreased turbidity at pH 3.5 is less clear. Assuming that this is approximately the pH of total charge neutralization, the possible formation of molecular association complexes may have resulted in a solubilization phenomenon and, consequently, decreased turbidity.

3A.3.3. The Stability of CM-chitin Turbidity

The stability of the measured turbidities due to CM-chitin was shown to be a function of both the CM-chitin concentration and the pH of the medium (Fig. 3A.3). The turbidities were most stable (for at least 20 min) when the CM-chitin concentration was low, *i.e.* up to 100 μg/ml, regardless of the pH. However, at the higher concentration of 500 μg/ml turbidities were stable only at pH 4.0 suggesting that the solubility of any association complexes that may have formed has been exceeded at this concentration. The instabilities in turbidity may be the result of a slow solubilization phenomenon due to a gradual association of the CM-chitin molecules at the higher concentration. Thus, standard calibration curves could be constructed at pH 2.5, 4.0 and 5.6 for CM-chitin concentrations up to 300 μg/ml (Fig. 3A.4). The correlation coefficients, r, determined from regression analysis at

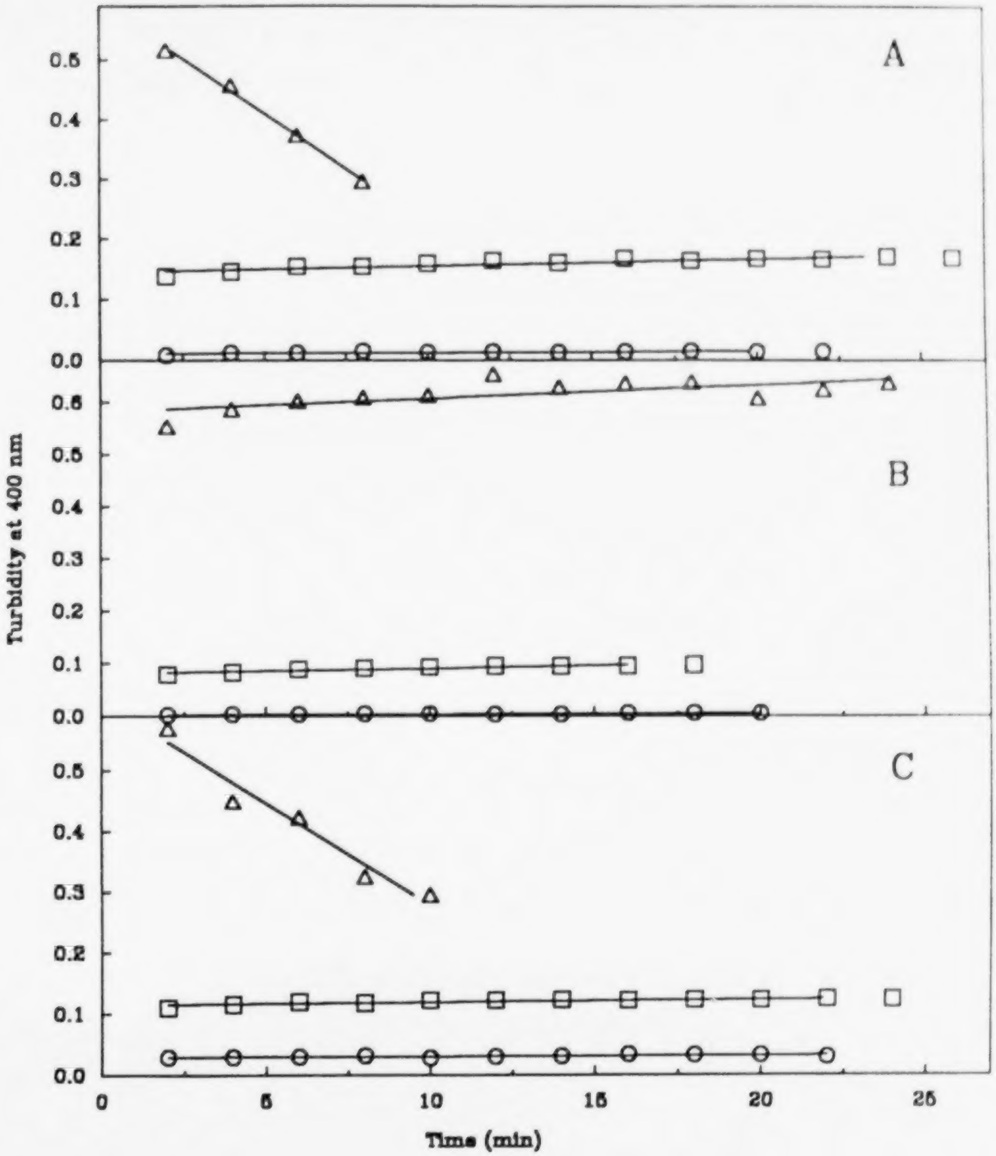

Fig. 3A.3. Stability of the CM-chitin turbidity as a function of the CM-chitin concentration at pH 5.6, 4.0, and 2.5 in 1:1 v/v isopropanol:acetate buffer solution. A. pH 5.6; B. pH 4.0; C. pH 2.5. ○ , 20 μg/ml; □ , 100 μg/ml; △ , 500 μg/ml. The ionic strength was 0.2.

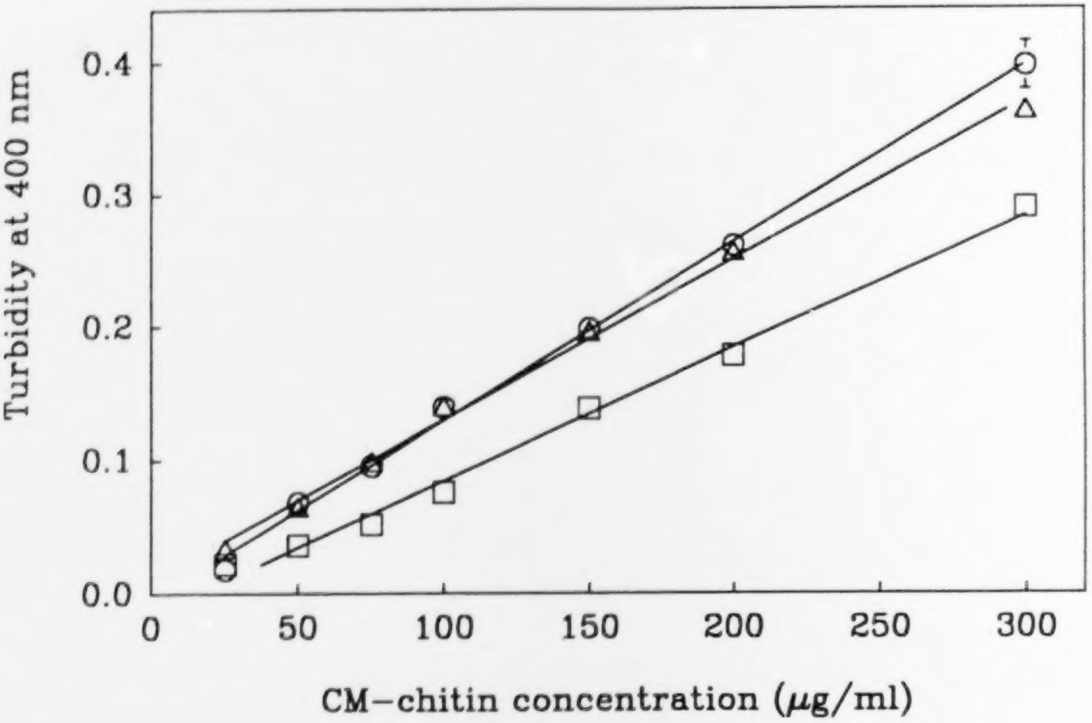

Fig. 3A.4. Standard calibration curve for the turbidimetric determination of CM-chitin in 1:1 v/v isopropanol:acetate buffer solution. ○ , pH 5.6, r = 0.999; □ , pH 4.0, r = 0.996; ▵ , pH 2.5, r = 0.998. Means ± SEM (n = 3) are shown.

the above pHs were 0.998, 0.996, and 0.999, respectively. The lower limit of detection was approximately 25 μg/ml of CM-chitin. If the sample concentration was below this limit, nephrometric method was suggested. Thus, when CM-chitin concentrations in liposome systems are greater than 300 μg/ml dilution of the sample should be made prior to mixing with the aqueous isopropanol solution to avoid aggregation into large particles.

3A.3.4. CM-chitin Turbidity at Different Ionic Strength

The effect of the ionic strength of the aqueous medium in the sample on the turbidity measurements can be seen in Fig. 3A.5 as a function of pH. Generally, there is a sharp increase in turbidity from 0.05 to about 0.15 ionic strength at pH 4.0 and 5.6 followed by relative insensitivity to ionic strength up to 0.5. In contrast, at pH 2.5 the sensitivity to ionic strength is only slight, up to 0.2, but becomes increasingly sensitive at higher ionic strengths. In all cases it would appear that increases in the ionic strength suppress the solubility of CM-chitin in the medium in a manner somewhat dependent on the pH, leading to increased turbidities. Thus, it would be important to control the ionic strength in these determinations, but if maintained at 0.15 to 0.3 the turbidities would be the least sensitive to ionic strength at each pH.

3A.3.5. Recovery of CM-chitin in the Presence of Lipids

Recoveries of CM-chitin and reproducibilities of the analyses using this

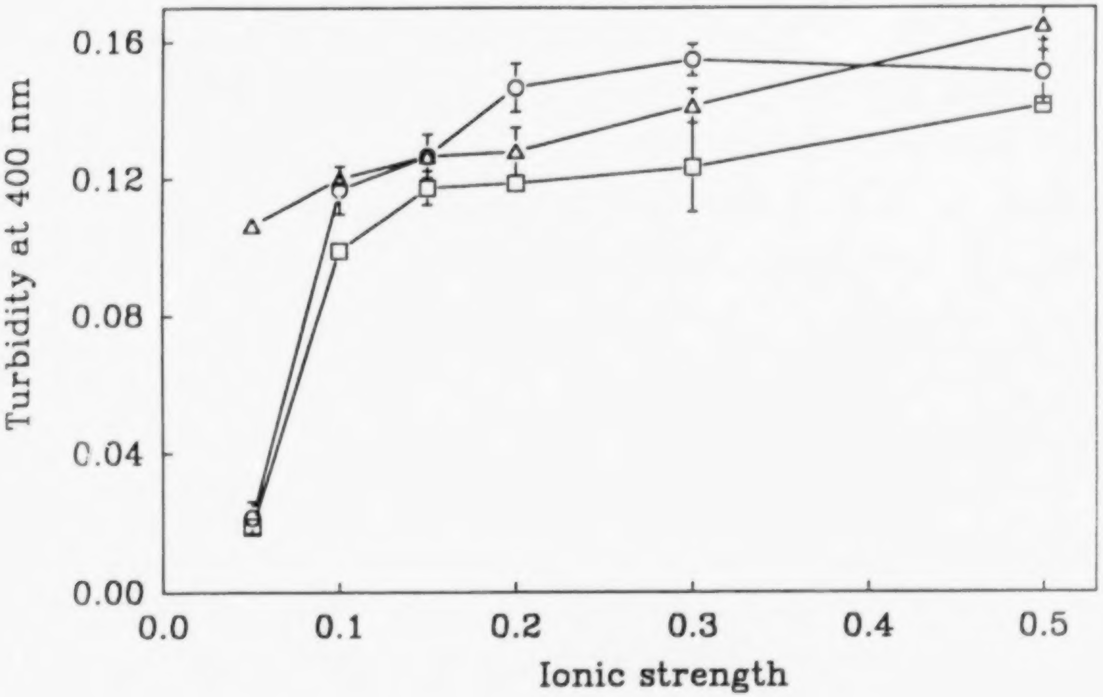

Fig. 3A.5. Dependence of CM-chitin turbidity on the ionic strength at pH 5.6, 4.0, and 2.5 in 1:1 v/v isopropanol:acetate buffer solution. ○ , pH 5.6; □ , pH 4.0; △ , pH 2.5. The concentration of CM-chitin was 100 μg/ml. Means ± SEM (n = 3) are shown.

technique are compared in Tables 3A.1 and 3A.2. Generally, the recoveries of CM-chitin from the analyses of CM-chitin-coated DPPC liposomes were excellent at each concentration examined, with the possible exception at pH 4.0 for an initial concentration of 200 μg/ml (F-test, p=0.05). It can be seen from analyses at the various pHs (R.T. ~ 22°C), that the recovery of CM-chitin from DMPC liposomes at pH 4.0 was significantly less than theoretical whereas recoveries from EPC liposomes at pH 5.6 and pH 2.5 were significantly greater than theoretical (F-test, p=0.05). Both DMPC and EPC liposomes are in the liquid crystalline state at this temperature therefore, the reduction in turbidity at pH 4.0 in DMPC systems and the increase in turbidity at pH 2.5 or 5.6 in EPC systems reflect the differences in the interactions between the phospholipid and CM-chitin molecules at the respective pHs.

3A.4. Summary

Since the determination of a polymer such as CM-chitin in a complex system of CM-chitin-coated liposomes in the presence or absence of drug would be difficult without interferences or requiring separations, the turbidimetric method as described here could offer a simple, rapid spectrophotometric means of determination. The method was applicable to the phospholipids DMPC, DPPC, and EPC using a 1:1 v/v isopropanol:acetate buffer solution but could be extended to other compositions providing a solvent system is found which dissolves the lipids but not CM-chitin. Also, in principle, the method could be applied to other polymer-coated liposome sytems as well.

Table 3A.1. Percent recovery of CM-chitin in the prescence DPPC as a function of pH and CM-chitin concentration.

pH	CM-chitin concentration (μg/ml)		
	50	100	200
5.6	99.9 (4.9)	103.7 (3.7)	98.5 (1.7)
4.0	92.1 (4.3)	98.6 (7.2)	114.1 (6.6)*
2.5	100.5 (6.3)	104.1 (3.6)	103.0 (2.4)

[a] Number in the brackets is the standard deviation of 6 measurements in acetate buffer:isopropanol (1:1 v/v) solution;

[b] F-test, comparsion with theratical value, $p=0.05$;

*Significant.

Table 3A.2. Percent recovery of CM-chitin at 100 μg/ml as a function of pH and phospholipid.

pH	DPPC	DMPC	EPC
5.6	103.7 (3.7)	101.0 (5.4)	112.2 (3.0)*
4.0	98.6 (7.2)	86.2 (9.3)*	97.5 (5.9)
2.5	104.1 (3.6)	102.7 (6.4)	115.3 (4.8)*

[a] Number in the brackets is the standard deviation of 6 measurements in acetate buffer:isopropanol (1:1 v/v) solution;

[b] F-test, comparsion with theratical value, $p=0.05$;

*Significant.

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CHAPTER 3

Part B

CM-CHITIN-COATED LIPOSOMES: PREPARATION, STABILITY AND RELEASE OF ACETYLSALICYLIC ACID[3]

3B.1. Introduction

The majority of liposome studies has involved the administration of liposomes intravenously, or intraperitoneally as drug carriers for the delivery of therapeutic or diagnostic agents to specific target tissues. However, there has been increasing interest in the potential use of liposomes for drug delivery by the oral route (*Woodley, 1985*). The main problems associated with orally-administered liposomes pertain to their relatively poor stabilities in the milieu of the GIT and, consequently, leakage of entrapped agents.

Many attempts have been made to enhance the stability of liposomes. Two major approaches, polymerized liposomes (*Regen et al., 1981 & 1985; Tirrell et al., 1985; Tackigawa and Tirrell, 1985; Ohno et al., 1987; Rinsdorf and Schlarb, 1988; Hub et al., 1990*) and polymer-coated liposomes (*Takada et al., 1984; Sunamoto et al., 1985, 1987 & 1988; Sato et al., 1986*), have been proposed thus far. For instance, *Hupfer and Ringsdorf (1981)* have successfully produced polymerized vesicles that withstand 50% ethanol dilution and dehydration procedures during sample preparation for scanning electron microscopy. *Tirrell et al. (1984)* have reported that coatings of poly(acrylic acid), poly(methacrylic acid) and poly(α-ethylacrylic acid) can be used to modify the properties and behavior of liposomes. *Kondo et al. (1983, 1984, 1985 & 1987)* and *Izawa et al., (1986)* have used carboxymethyl chitin to produce stable artificial red blood cells (ARBC) and *Sunamoto et al. (1983)* have used other specialized polysaccharides to increase the stability of liposomes and decrease their leakage of entrapped solute after intravenous administration. Subsequently, these same workers tested *O*-palmitoylpullulan- and *O*-palmitoylamylopectin- coated egg phosphatidycholine liposomes, where the polymers were added to preformed small unilamellar vesicles, for 6-carboxyfluorescein release in the presence of bile salts at pH 8.6 and found increased release of the marker (*O'Connor et al., 1985*). This unexpected result was, perhaps, due to an unfortunate choice of composition and conditions as will be demonstrated from the results of the present study.

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[3] A version of this part has been accepted by *J. Control. Rel.* (in press).

The aim of this work was to determine the optimum conditions for coating liposomes with the polysaccharide, carboxymethylchitin (CM-chitin), which bestows increased stability of the liposomes in sodium cholate solutions and decreased release of the model water-soluble drug molecule, acetylsalicylic acid (ASA). In this regard, the coating efficiency of a fully-saturated, synthetic phospholipid liposome by CM-chitin, comparison of methods of preparation, the encapsulation efficiency of ASA, and the kinetics of release of ASA from the liposomes have been determined.

Particular attention was given to selecting liposomes in the gel phase and at a pH at which CM-chitin has been found to be stable.

3B.2. Materials and Methods

3B.2.1. Materials

Dipalmitoylphosphatidycholine (DPPC) was purchased from Sigma Chemical Co. Acetylsalicylic acid and sodium cholate from Aldrich Chemical Co. were used as received. Carboxymethyl chitin ($pk_a = 3.5$; viscosity of 1% solution = 14 cps) was a gift from Nanyo Kasei Co., Japan. All other chemicals were reagent grade, and water was twice-distilled.

3B.2.2. Preparation Methods

Two methods were used to prepare CM-chitin-coated liposomes. One method involved adding CM-chitin during liposome preparation as follows: Twenty-five mg of DPPC in 3 ml of dichloromethane were combined with 1 ml of pH 7.0 (0.085 M) phosphate buffer, then sonicated (*Model W-375 Sonicator, Heat System-Ultrasonics, Inc., Cup-horn, Model 431*) at 10 Khz for 4 min to form a w/o emulsion. The emulsion was diluted with 1 ml of CM-chitin aqueous solution then vortex-mixed to form a w/o/w emulsion. Subsequently, the organic solvent was slowly removed under reduced pressure by rotary evaporation resulting in CM-chitin-coated liposomes. When ASA (30 mM) was included it was incorporated together with the phospholipid in the organic solvent. The other method involved preparing liposomes as above, except in the absence of CM-chitin, then diluting the liposome preparation (2 ml) with CM-chitin solution (1 ml) followed by incubation at room temperature for about one hour.

3B.2.3. Microscopic Observation

A drop of liposomes or polymer-coated liposomes was placed on a slide with coverslip and observed at 1000× magnification using a *Zeiss Standard 14* microscope fitted with a 35 mm camera. Individual frames were exposed for 4 sec using *Kodak Pan X film (100 ASA).*

3B.2.4. Particle Size Determination

Liposomes were prepared as described, then treated by gel filtration chromatography (*Sephadex G-25, 1.6 × 70 cm*) to remove free CM-chitin using pH 5.6 acetate buffer as the eluting solvent. Liposome fractions were pooled and diluted with the same buffer (20 μl to 3 ml of buffer) and sized by laser light scattering (*Brookhaven Particle Sizer, Model BI-90*) as follows: 2500 cycles/run, 4 runs for each of triplicate samples at a count rate of about 27 kcps.

3B.2.5. ASA Encapsulation Efficiency

The encapsulated ASA was separated from free ASA by centrifugation (*Beckman Model L8-55 ultracentrifuge*) at 135,000 × g for 20 min. The pellet containing entrapped ASA was dissolved in chloroform:isopropanol (1:4v/v) to 10 ml then analyzed for ASA by HPLC as follows: solvent delivery system (*Waters, Model 501*), UV detector at 280 nm (*Waters, Lambda-Max, Model 481*), Rheodyne injector (*Spectra Physics, Model 7125*), 20 μl injection loop, C_{18} reverse phase chromatography column (*Waters, 15-cm Novapak*, 5 μm particle size), integrator-plotter (*Waters, Model 740*). The mobile phase was 20% acetonitrile aqueous solution containing 0.1% phosphoric acid at pH 2.7 delivered at a flow rate of 1.5 ml/min.

3B.2.6. CM-chitin Coating Efficiency

The CM-chitin coating efficiency was determined as described in 3A. Briefly, CM-chitin-coated liposomes were separated from free CM-chitin by gel filtration (*Sephadex G-25, 1.6 × 70 cm*) using pH 5.6 acetate buffer as the eluting solvent; eluted fractions of the coated liposomes were collected and pooled. After allowing sedimentation of the polymer-coated liposomes for 12 hr at 8°C, the supernatant was removed by Pasteur pipette then the sediment was diluted to 10 ml with buffer solution. Subsequently, 5 ml were removed, mixed with 5 ml of isopropanol and the turbidity was measured at 400 nm. The coating efficiency was calculated as the ratio of the weight of associated CM-chitin (mg) per 100 mg of DPPC.

3B.2.7. Stability in Sodium Cholate Solutions

One-ml samples of either uncoated or CM-chitin-coated liposomes were mixed with different volumes of 20 mM sodium cholate solution, then diluted to 10 ml with pH 5.6 acetate buffer at room temperature (22°C). The turbidity of the mixture was measured spectrophotometrically at 400 nm. The relative stabilities of the liposomes were assessed by the equilibrium turbidity changes as a function of the added sodium cholate concentrations.

3B.2.8. ASA Release Studies

CM-chitin-coated liposomes containing ASA were prepared as described, then treated by gel permeation chromatography (*Sephadex G-25, 1.6 × 70 cm*) to remove free ASA using pH 5.6 acetate buffer as the eluting solvent. Liposome fractions were pooled then incubated at 37°C (*Dubnoff Metabolic Shaker*) following which aliquots (1 ml) of the thoroughly-mixed preparation were removed at 20 min intervals, centrifuged, and the supernatants were analyzed by HPLC. The following was used to calculate the release of ASA:

$$\text{ASA release (\%)} = (C - C_o)/(C_t - C_o) \times 100$$

where C_o is the initial ASA concentration in the supernatant before incubation, C_t the total ASA and SA concentrations in the liposome suspension, and C the ASA and SA concentrations in the sample supernatant after time t.

3B.3. Results and Discussion

3B.3.1. Morphology of CM-chitin-Coated Liposomes

The particle size of the liposome preparations was estimated to be in the range of 1.3 to 8.9 μm ($p=0.1$) by laser light scattering. CM-chitin-coated liposomes, coated after liposome formation, resulted in increased average diameters of liposomes ranging from 4.16 μm (control liposomes) to 4.56 μm while liposomes coated with CM-chitin during liposome formation had smaller diameters than the control liposomes (Table 3B.1), suggesting different mechanisms of CM-chitin coating. CM-chitin coatings formed after liposome formation were based on hydrophilic adsorption at the liposome surfaces, thus, the liposome core and the CM-chitin coating determined the size of the particle. In contrast, CM-chitin coating formed during liposome formation not only covered the surfaces of the liposomes but also participated in the formation of liposomes. In the other words, CM-chitin mixed with and became anchored to the bilayers causing a condensation of the phospholipid molecules in the liposomes resulting in a smaller overall size. It can be observed from the data in Table 3B.2 that this was a concentration-dependent effect.

Optical microscopic inspection of individual particles clearly revealed the multilamellar structure of the control (uncoated) liposomes (Fig. 3B.1A) whereas this was obscure for the CM-chitin-coated liposomes (Fig. 3B.1B). In the latter case the particles were aggregated and appeared to be enclosed by a polymer shell.

The results of the gel permeation technique to remove free ASA from liposomal ASA are illustrated in Fig. 3B.2. Under these conditions the liposomes were eluted in the void volume as fractions 8-15 and the free ASA was eluted in fractions 15-26, approximately. Liposome fractions were turbid while ASA had no light absorption at 400 nm. Likewise, liposomal components in isopropanol solution were shown not to interfere with the UV absorption of ASA at 276 nm. This elution

Table 3B.1. Particle size range of CM-chitin-coated liposomes as a function of liposome preparation methods.

CM-chitin added	Average diameter μm, (STD)	Diameter range (μm, p=0.1)
0	4.16 (0.25)	1.35 ~ 7.96
1% CM-chitin added during liposome formation	3.92 (0.19)	1.29 ~ 7.47
1% CM-chitin added to preformed liposomes	4.59 (0.69)	1.42 ~ 8.87

Table 3B.2. Particle size range of CM-chitin-coated liposomes as a function of CM-chitin concentration.

CM-chitin concentration (%)	Average diameter μm (STD)	Diameter range (μm, p=0.1)
0	4.16 (0.25)	1.35 ~ 7.96
0.5	4.58 (0.38)	1.55 ~ 8.62
1.0	3.92 (0.19)	1.29 ~ 7.47
1.5	3.80 (0.22)	1.92 ~ 6.13
2.0	3.32 (0.43)	1.34 ~ 5.91

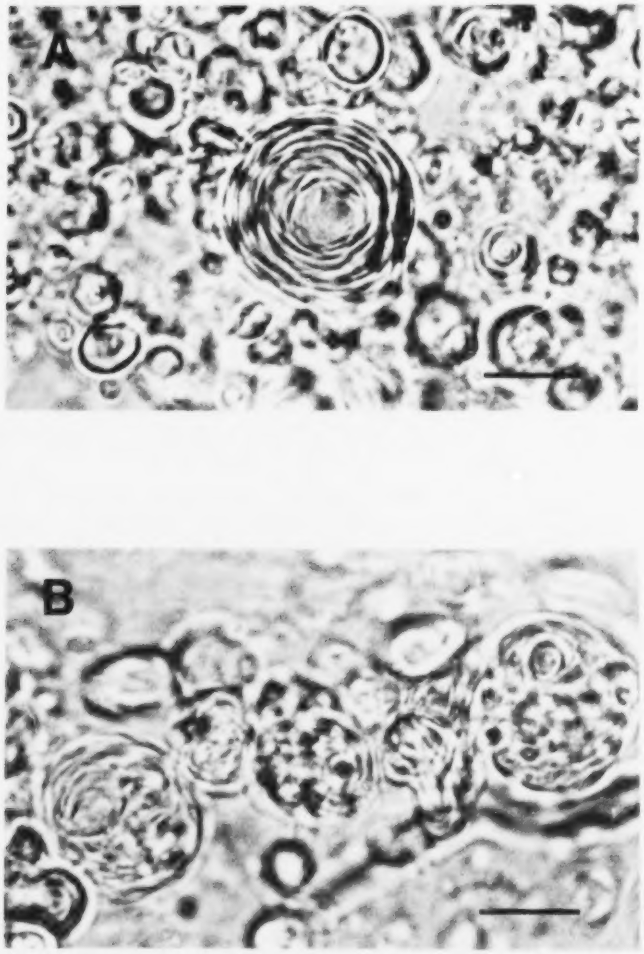

Fig. 3B.1. Micrographs from optical microscope showing the microstructure of uncoated and CM-chitin-coated DPPC liposomes. A) Uncoated liposomes, B) 1.0% CM-chitin-coated liposomes coated during liposome preparation. Bar length = 10 μm.

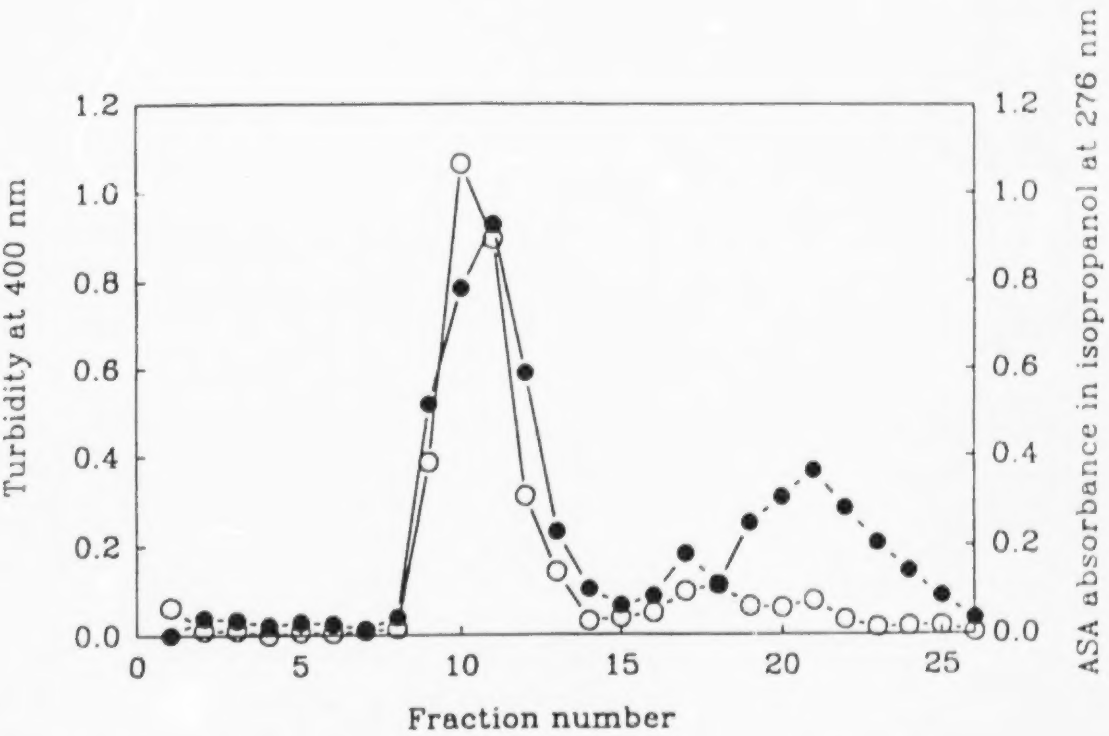

Fig. 3B.2. Profile of the separation of entrapped ASA in DPPC liposomes from unentrapped ASA by gel permeation chromatography, Sephadex G-25, 1.6 X 70 cm column, pH 5.6 sodium acetate buffer. ○ , turbidity at 400 nm; ●, ASA absorbance in isopropanol at 276 nm.

pattern was reproducible for either uncoated or CM-chitin-coated liposomes demonstrating application of gel permeation chromatography to polymer-coated liposomes.

3B.3.2. ASA Encapsulation Efficiency

The encapsulation efficiency of ASA in uncoated and CM-chitin-coated DPPC liposomes is described in Table 3B.3 as a function of the initial ASA concentration. The encapsulation efficiency gradually increased with ASA concentration up to 60 mM, but then increased dramatically at higher concentrations, being approximately 20-fold greater following an 8-fold increase in the initial ASA concentration. It is also apparent that the CM-chitin coating of liposomes did not significantly influence the ASA encapsulation efficiency.

3B.3.3. CM-chitin Coating Efficiency

The coating efficiency of liposomes by CM-chitin was dependent on the initial CM-chitin concentration and the coating procedure. The results, illustrated in Fig. 3B.3, indicate the differences in coating efficiency at various initial CM-chitin concentrations up to 2 percent. When the CM-chitin was added during liposome formation more of the polymer became associated with liposomes at initial CM-chitin concentrations up to 1.5 percent compared to when the polymer was added to preformed liposomes. This is not surprising since the CM-chitin molecules were intimately in contact with all of the bilayers comprising the multilamellar liposome

Table 3B.3. Comparison of ASA encapsulation efficiency in uncoated and 1.0% CM-chitin-coated DPPC liposomes. Number in brackets is the standard deviation of triplicate measurements with a DPPC concentration of 74 mM.

Initial ASA Concentration (mM)	Encapsulation efficiency (ASA:DPPC mole ratio %)	
	Uncoated liposomes	Coated liposomes
15	1.5 (0.2)	1.5 (0.1)
30	2.2 (0.1)	2.3 (0.1)
60	4.8 (0.1)	5.1 (0.7)
90	18.6 (0.6)	18.1 (2.2)
120	27.2 (1.7)	24.6 (8.2)

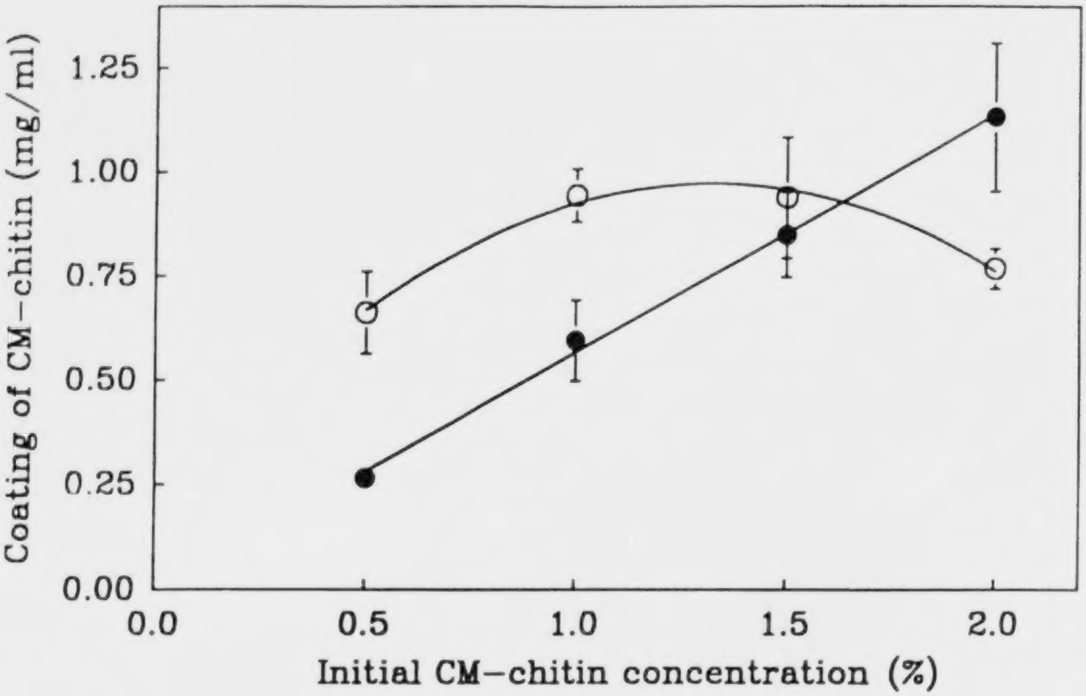

Fig. 3B.3. CM-chitin coating efficiency as a function of initial CM-chitin concentration and coating method. Experiments were conducted at room temperature (~22°C) at a DPPC concentration of 74 mM. ○ , liposomes coated during liposome formation; ● , coated preformed liposomes. ± SD (n=3).

structure when added during liposome formation whereas only the outer bilayers of the liposomes were available for interaction with the CM-chitin molecules when added to preformed liposomes. In the former case, the coating efficiency rose only slightly with additional CM-chitin, reached a maximum, then actually decreased when 2 percent CM-chitin was added. This suggests a capacity-limited interaction process which would occur if the CM-chitin molecules interdigitated the ordered DPPC molecules in the bilayers. On the other hand, in the latter case, the coating of preformed liposomes by CM-chitin is proportional to the CM-chitin added which indicates surface binding. Since the coating efficiency increased linearly up to 2 percent of added CM-chitin, the surface film formed is probably multimolecular. The chemical structure of CM-chitin (Fig. 3B.4) would suggest that it would undergo both hydrophobic interaction in the hydrocarbon region of the bilayers as well as polar group interactions which could have the effect of altering the permeability to entrapped solutes.

3B.3.4. Liposome Stability Studies

The degree of physical stability of CM-chitin-coated liposomes in sodium cholate solution was also a function of the liposome coating procedure. Fig. 3B.5 shows uncoated liposomes to be unstable in 2 mM sodium cholate solution as expected. However, each of the CM-chitin-coated liposome preparations was stable in sodium cholate solutions as high as 3-4 mM. In comparison, only about 65% of the original turbidity of the uncoated liposomes remained after addition of 2 mM of

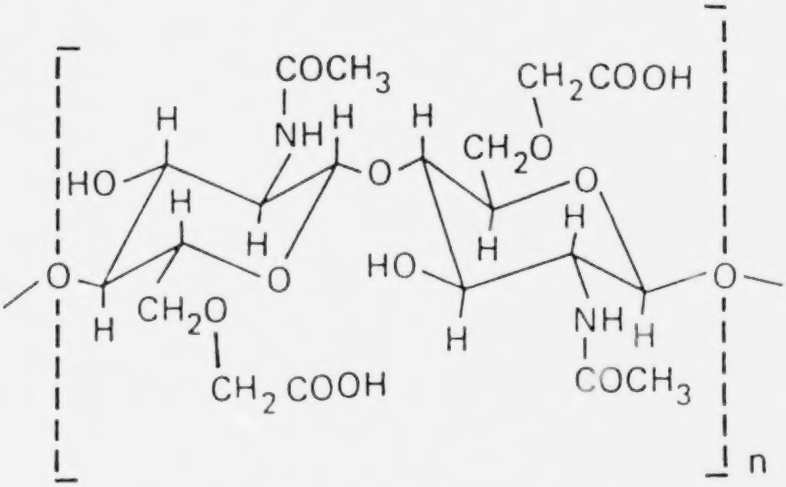
COCH3
NH
H
HO
O
CH2O
CH2COOH
HO
NH
COCH3
n

Fig. 3B.4. Chemical structure of carboxymethyl chitin.

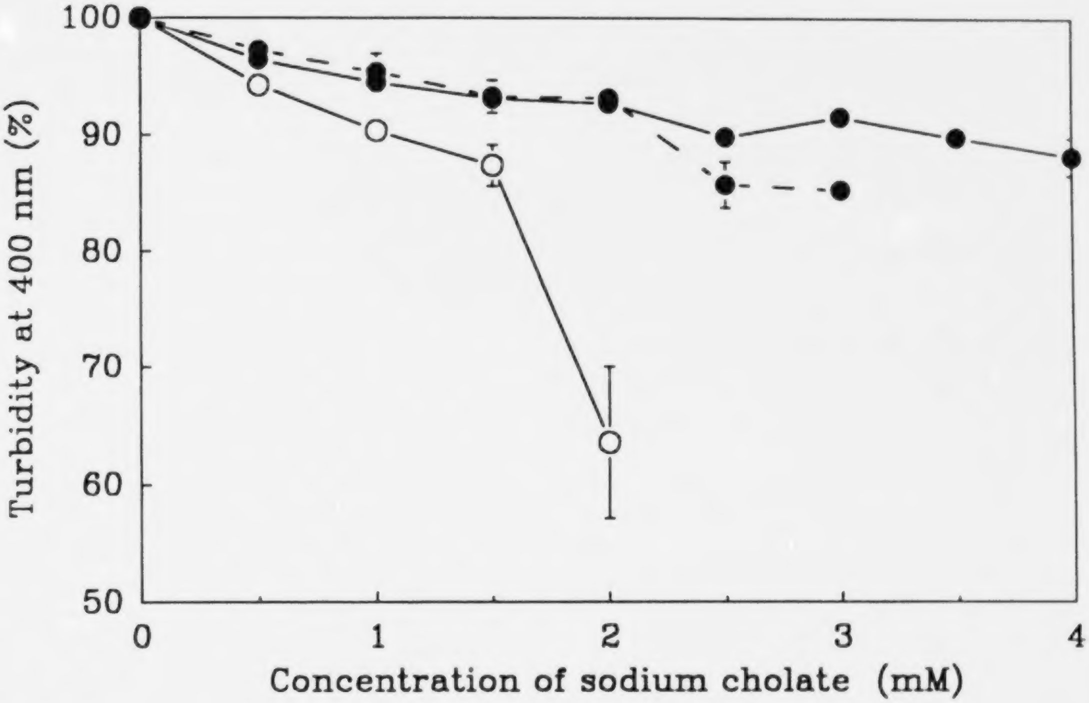

Fig. 3B.5. Turbidity of CM-chitin-coated and uncoated DPPC liposomes after dilution in sodium cholate solution as a function of coating procedure, in pH 5.6 acetate buffer at 22°C. ○ ——— ○ , uncoated liposomes; ● --- ● , 1.0% CM-chitin added to preformed liposomes; ● ——— ● , 1.0% CM-chitin added during liposome formation. ± SD (n=3).

sodium cholate solution whereas more than 90% of the original turbidity of 1% CM-chitin-coated liposomes, coated during liposome formation, remained after dilution with 3 mM sodium cholate solution and about 85% for the preformed liposomes. Statistically, the stability between CM-chitin-coated and uncoated liposomes was significant especially at 2 mM sodium cholate concentration (F-test, $p=0.05$), however, there was no significant difference between two procedures.

Fig. 3B.6 illustrates that the stability of liposomes, coated during liposome formation, was also a function of the initial CM-chitin concentration used to coat the liposomes. It is apparent that after the addition of 0.5% CM-chitin, the coated liposomes became stable at 2 mM but not at 2.5 mM sodium cholate concentration. Increasing the initial CM-chitin concentration to 1.0% produced even greater stability of the liposomes and were stable even in the presence of 3 mM sodium cholate solution (F-test, $p=0.05$). Furthermore, increasing the initial CM-chitin concentration to 2.0% appeared to yield similar, although slightly lower liposome stability at sodium cholate concentrations greater than 2mM. The stability results show a reasonable correlation with the measured CM-chitin coating efficiencies since liposomes coated during liposome formation from a 1.0% initial CM-chitin concentration yielded the greatest coating efficiency (Fig. 3B.3). The results at 2% CM-chitin concentration point towards reduced coating efficiency and stability of the liposomes, suggesting a possible increased perturbation of the lipid bilayers at the higher CM-chitin concentration.

The turbidimetric method, also used by others (*Regen et al., 1980*) to study

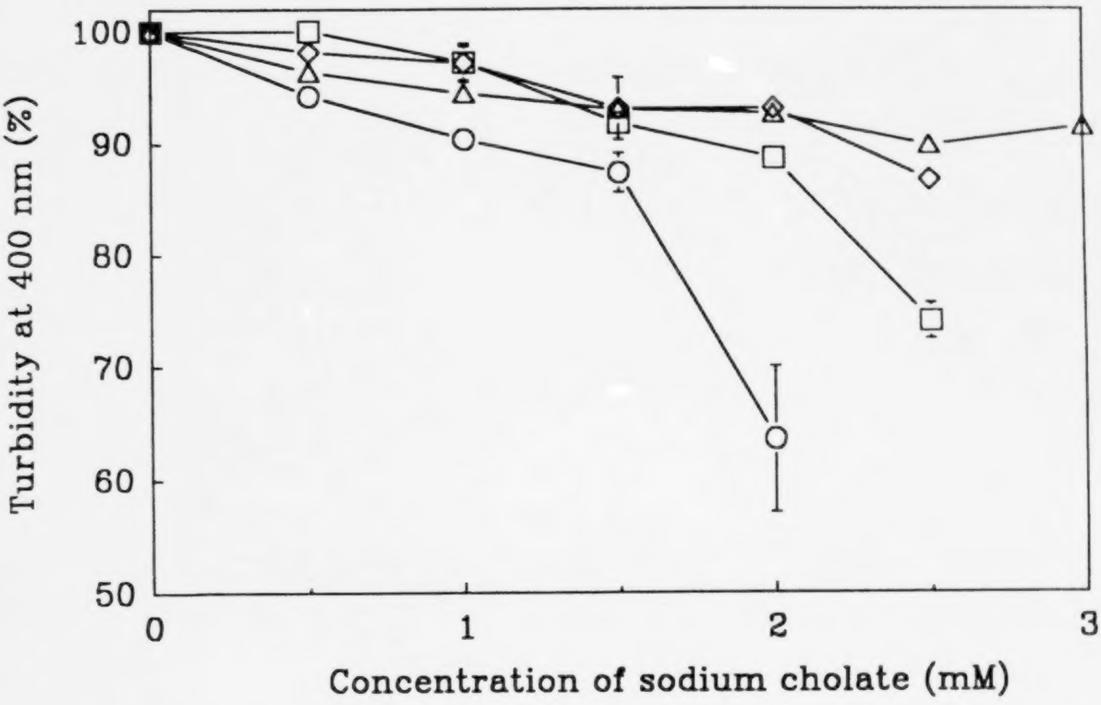

Fig. 3B.6. Turbidity of CM-chitin-coated and uncoated DPPC liposomes as a function of initial CM-chitin concentration after dilution in sodium cholate solution . ○ , uncoated liposomes; □ , 0.5% CM-chitin-coated liposomes; △ ,1.0% CM-chitin-coated liposomes; ◇ , 2.0% CM-chitin-coated liposomes. ± SD (n=3).

the stability of polymerized liposomes in ethanol solutions, supports the use of a 400 nm wavelength. In this study, the same principle was used without interference by sodium cholate and the other solutes in the system. Likewise, CM-chitin-coated egg phosphatidycholine (EPC) liposomes were demonstrated to be more stable against the detergent effect of sodium 1-dodecyl sulfate using a turbidimetric method (*Kato and Kondo, 1987*). Since sodium cholate is prevalent as an endogenous detergent in the GI tract, solutions of it represent a more appropriate vehicle for the demonstration of liposome stability following oral administration. Bile salt damage of liposomes, like other detergents, requires a critical detergent:lipid ratio before mixed micelles begin to form and solubilization of the liposomes occurs (*Lichtenberg, 1985; Rogers et al., 1990*). Attainment of this critical ratio is a function of the detergent concentration and the intermolecular energies of the lipid molecules in the liposomal bilayers. The latter is determined by the fluidity of the hydrocarbon chains and the integrity of the outer hydrated polar group region. The inclusion of additives which increase hydrocarbon chain separation and decrease the surface energy barrier will result in a reduction of the detergent:lipid ratio for mixed micelle formation. Thus, the coating of egg phosphatidylcholine liposomes in the liquid crystalline state by *O*-palmitoylpullulan or *O*-palmitoylamylopectin at pH 8.6 as reported by *O'Connor et al. (1985)* would be expected to decrease interactions between the lipid molecules in the bilayers, thereby increasing the ease with which detergents can participate with the lipid molecules in mixed micelle formation, leading towards increased permeability of the 6-carboxyfluorescein marker. In contrast, it is concluded that the interaction of

CM-chitin with gel phase DPPC liposomes at pH 5.6 contributes to the coherent structure of the bilayer membranes but only over a narrow concentration range (approx. 0.5%-1%) giving a polymer-coated liposome structure which is more stable against sodium cholate than uncoated liposomes. This behavior of CM-chitin occurs whether the polymer is included during liposome formation or added to preformed liposomes, but the magnitude of the effect differs slightly as shown in Fig. 3B.5, particularly in sodium cholate solutions greater than 2 mM.

3B.3.5. ASA Release Studies

The release of encapsulated ASA from uncoated and CM-chitin-coated DPPC liposomes in pH 5.6 buffer at 37°C is described in Fig. 3B.7. ASA was rapidly released from uncoated DPPC liposomes, about 85% being released within 20 min and the remaining being gradually released over a period of an hour. In contrast, CM-chitin-coated liposomes yielded decreased release of ASA, to the extent of about 25% within 20 min from liposomes coated by CM-chitin during liposome formation, or 70% in 20 min from preformed liposomes coated with CM-chitin, the release of the remaining ASA was so slow that there was not significant amount released for two hours, however, the extent of ASA release was significantly different among these three formulations (F-test, $p=0.05$). These different results are interpreted to mean that the manner and extent to which CM-chitin is associated with the liposomes plays an important role in the degree of cohesiveness and continuity of the bilayers in the liposome structure and in the polymer film as a physical barrier towards diffusing

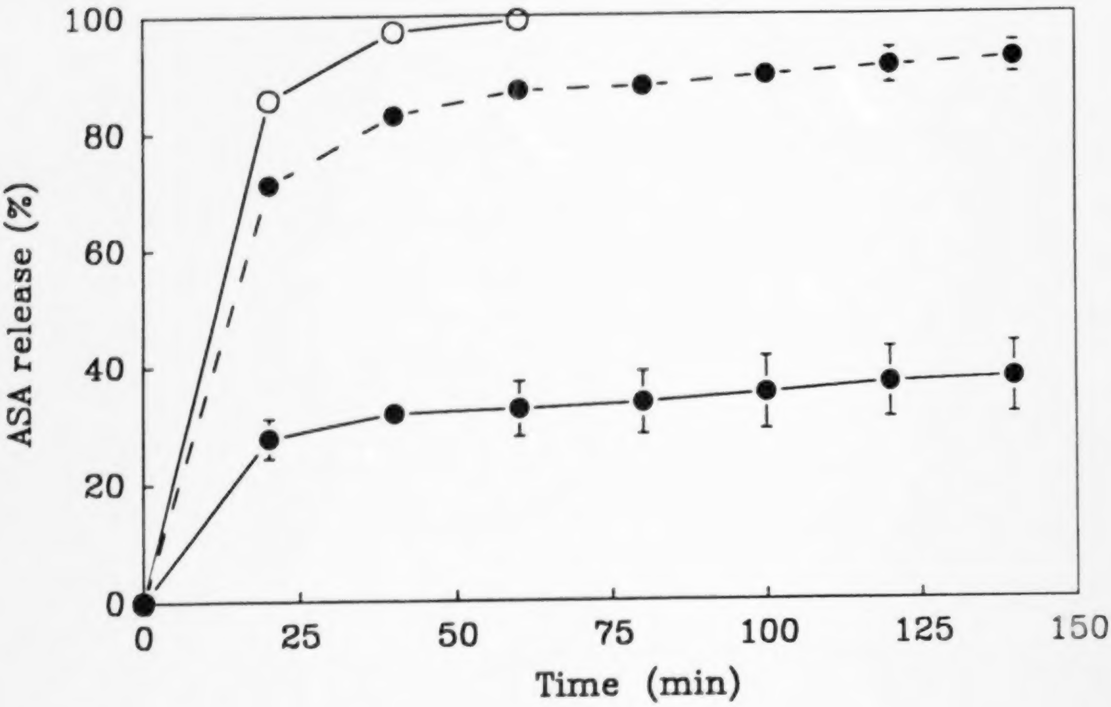

Fig. 3B.7. The release of ASA from ASA-encapsulated CM-chitin-coated and uncoated DPPC liposomes as a function of the coating procedure in pH 5.6 acetate buffer at 37°C. ○ ——— ○ , uncoated liposomes; ● --- ● , 1.0% CM-chitin added to preformed liposomes; ● ——— ● , 1.0% CM-chitin added during liposome formation.

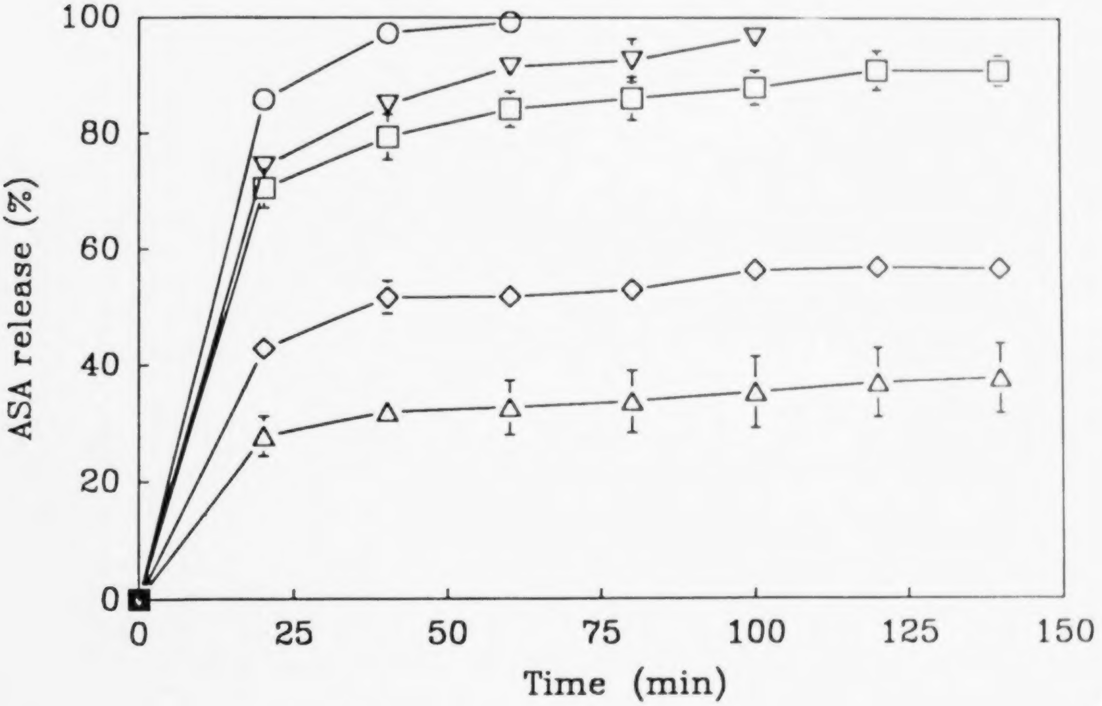

Fig. 3B.8. The release of ASA from ASA-encapsulated CM-chitin-coated (during liposome formation) and uncoated DPPC liposomes as a function of CM-chitin concentration in pH 5.6 acetate buffer at 37°C. ○ , uncoated liposomes; □ , 0.5% CM-chitin-coated liposomes; ▵ , 1.0% CM-chitin-coated liposomes; ◇ , 1.5% CM-chitin-coated liposomes; ▿ , 2.0% CM-chitin-coated liposomes. ± SD (n=3).

solute. Since the release was carried out close to the DPPC phase transition temperature (42°C), CM-chitin added during liposome formation was at least partially located in the bilayers and could have increased the ordered structure of the bilayers (with decreased fluidity). This would result in a decreased permeability and leakage of ASA.

The release of ASA from CM-chitin-coated DPPC liposomes, coated during liposome formation, was also influenced by the initial CM-chitin concentration as shown in Fig. 3B.8. At a CM-chitin concentration of 0.5%, the release of ASA after 50 min was reduced by about 20%. At 1% levels, the release of ASA was reduced approximately 3-fold. This compares with the decreased release of carboxyfluorescein (CF) from EPC liposomes using derivatized pullulan (*Sunamoto et al., 1987*). However, at higher CM-chitin concentrations a surprising reversal took place. Thus, at 1.5% the release of ASA was reduced approximately 2-fold only whereas at 2% CM-chitin concentration the release of ASA was hardly 10% less than that from uncoated liposomes. Thus, it would appear that the main effect of polymer coating on drug release is the influence which the polymer exerts on the bilayer structure of the liposomes rather than the polymer film acting as a barrier to ASA release. Over a narrow range of concentration of polymer added during liposome formation, the barrier to the transport of solute increases whereas larger amounts of added polymer lead to a reduction of the barrier function of the bilayers to transport.

3B.4. Summary

The results of this study demonstrate a clear effect of the coating of DPPC liposomes by CM-chitin on liposome stability and the release of ASA from CM-chitin-coated liposomes. The effects produced were somewhat dependent on the coating procedure. Adding CM-chitin during liposome formation enabled the polymer to become associated with the surfaces of each of the bilayers in the multilamellar structure whereas adding the polymer to preformed liposomes only coated the outer bilayers and had a smaller influence on the release of encapsulated drug. The main significance of these studies is the possibility of developing a liposome system which possesses considerably greater stability in sodium cholate solutions then uncoated liposomes and which the leakage of entrapped drug, such as ASA, was at a much lower level by appropriate selection of the polymer concentration and the method used to form polymer-coated liposomes. This could have important implications in the design of a liposome drug delivery system for oral administration.

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CHAPTER 4

ACACIA-GELATIN-MICROENCAPSULATED LIPOSOMES: PREPARATION, STABILITY AND RELEASE OF ACETYLSALICYLICACID[4]

4.1. Introduction

The development of liposomes as drug delivery systems is often halted because of poor stability in the biological environment in which they are intended to be administered. The oral route in particular has not been successful due to hydrolytic influences of enzymes and pH and the solubilizing action of the bile salts. Attempts to improve liposome stability have used polymerized liposomes (*Regen, 1987*), polymer-encased liposomes (*Fukuda et al., 1986*), polymer-coated liposomes (*Ringsdorf and Schlarb, 1988; Sunamoto et al., 1983; Tirrell et al., 1985*), or microencapsulated liposomes (*Kibat et al., 1990; Nixon and Yeung, 1989; Yeung and Nixon, 1988*). In addition, controlled release of protein (*Ozden and Hasirci, 1990*) and sustained release of an ionized drug molecule from these modified liposomes have been demonstrated.

Microencapsulation of liposomes by polymers may have the advantage of stabilizing liposomes against hydrolytic influences in the gastrointestinal tract more efficiently than polymer-coated liposomes because of thicker shells which can be produced. Although synthetic polymers, such as the polyamides (*Nixon and*

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[4] A version of this chapter has been submitted for publication to *Pharm. Res.*

Yeung, 1989; Yeung and Nixon, 1988) may be suitable in some circumstances, nontoxic naturally-occurring polysaccharides are more appropriate for oral liposomes with which it is also possible to microencapsulate liposomes without the use of organic solvents.

This chapter describes the preparation of microencapsulated liposomes and the effect of this process on the encapsulation of an ionic solute and its release behavior. In addition, the degree of stability of the microencapsulated liposomes compared to the liposomes themselves in bile salt solutions has been measured. These properties of microencapsulated liposomes have been compared to similar properties previously observed with other polymer-coated liposomes.

4.2. Materials and Methods

4.2.1. Materials

L-α-Dipalmitoylphosphatidylcholine (DPPC) and gelatin (300 bloom, type A) were obtained from Sigma Chemical Co., St. Louis, MO. Acacia, formaldehyde (Fisher Scientific Co., Fairlawn, NJ), acetylsalicylic acid (ASA) and sodium cholate (Aldrich Chemical Co., Inc., Milwaukee, WI) were used as received. All other chemicals were at least reagent grade and deionized distilled water was used throughout.

4.2.2. Microencapsulated Liposomes by Complex Coacervation

Multilamellar liposomes (MLVs) were prepared by a simple hydration method. Typically, DPPC (34 μmol) and ASA (30 μmol) dissolved in 5 ml of chloroform were dried as a film in a round-bottom flask by rotary evaporation at 50°C then flushed with N_2 and left overnight in a vacuum oven at 30°C. Subsequently, the film was hydrated with 1 ml of pH 5.6 buffer solution (15 mM acetic acid + 157 mM sodium acetate) at 50°C and dispersed by vortex-mixing. MLVs (1 ml) were mixed with equal volumes (0.5 ml, 1.5 ~ 5% w/v in each case) of acacia and gelatin solutions then the preparation was adjusted to a pH of 4.0 using a pH 2.5 acetate buffer. The mixture was cooled to 8°C for 10 min to form microencapsulated liposomes. The microcapsule shell was hardened by the addition of 37% formaldehyde (1 ml) and the excess formaldehyde was removed by centrifugation and washing with pH 5.6 buffer solution.

4.2.3. Electron Microscopic Studies

a) *Negative staining*. The procedure for negative staining of a sample liposome preparation was as follows: a drop of liposome preparation was applied to a formvar film-coated copper grid and the excess was removed by filter paper. Phosphotungstic acid solution (1%, pH 7.0) was dropped onto the grid and after 10 sec it was removed with filter paper. The stained samples were examined in a Hitachi H-7000 electron microscope at 75 KV.

b) *Ultrathin section technique*. Liposomes were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer solution at pH 7.5 for 2 h. After three times rinsing with the buffer solution samples were further fixed with

1% osmium tetroxide in the same buffer solution for 2 h. After rinsing with water to remove excess osmium tetroxide 1% uranyl acetate was added and the sample left overnight. Subsequently, after rinsing with distilled water the samples were dehydrated with successive 10 min washings of acetone solutions (50%, 70%, 95%, and 2×100%). This was followed by successive 30 min washings of acetone:Spurrs' medium[5] (1:1 v/v, 1:3 v/v) then Spurrs' medium (twice for 1 h). The specimens were placed in flat embedding moulds containing Spurrs' medium and put in an oven at 70°C to polymerize for 8 h. Thin sections of approximately 60 ~ 90 nm were cut from the sample with a glass knife on an ultramicrotome and collected on 400 mesh copper grids. Post-staining was carried out with 2% uranyl acetate then the specimens were examined under the electron microscope.

4.2.4. Particle Sizing and Wall Thickness Determinations

Liposome preparations (20 μl) were diluted with pH 4.0 acetate buffer solution (3 ml) and sized by dynamic light scattering (*Brookhaven Instruments Particle Sizer, Model BI-90*) using a protocol of 2500 cycles/run, 5 runs for each of triplicate samples at a count rate of ca. 18 kcps. Wall thicknesses of microcapsules were estimated from the differences in mean size between microencapsulated and control liposomes.

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[5] Spurrs' medium consists of 23.6% v/v 4-vinylcyclohexene dioxide (ERL 4206), 14.2% v/v diglycidyl ether of polypropylene glycol (DER 736), 61.3% v/v nonenyl succinic anhydride (NSA) and 0.9% v/v dimethylaminoethanol (DMAE).

4.2.5. Analysis of Encapsulated ASA

Either liposome-encapsulated ASA or microencapsulated liposomal ASA was determined by centrifugation (135,000 x g for 10 min, *Beckman Model L8-55 Ultracentrifuge*) and removal of the supernatant. The pellet was then dissolved in chloroform:isopropanol solution (1:4 v/v) and analyzed by reverse phase HPLC (*Waters*) with spectrophotometric detection at 280 nm. The mobile phase was 20% acetonitrile at pH 2.7 (0.1% phosphoric acid) at a flow rate of 1.5 ml/min.

4.2.6. Liposome Stability Studies

A 1-ml aliquot of either control or microencapsulated liposomes was mixed with different volumes of 20 mM sodium cholate solution buffered to pH 5.6 then diluted to 10 ml with buffer at room temperature. Turbidities were monitored spectrophotometrically at 400 nm (*Regen et al., 1980*) (*Beckman spectrophotometer, Model 25*). Decreased turbidities were considered indicative of decreased stability (*Nagata et al., 1990; Roda et al., 1990*). Some studies were also conducted in pH 7.0 buffer solution (50 mM NaH_2PO_4, 35 mM NaOH).

4.2.7. Drug Release Studies

Microencapsulated liposomes containing ASA (0.24mmol) at pH 5.6 were incubated (*Dubnoff Metabolic Shaker*) with gentle agitation either at room temperature (23°C) or 37°C. At 20 min intervals 1 ml aliquots were withdrawn and centrifuged. The amount of ASA and SA in the supernatant was analyzed as before. The fraction of ASA released was calculated from,

$$\% \text{ ASA released} = (C-C_0)/(C_t-C_0) \times 100$$

where C_0 is the initial ASA concentration in the aqueous medium before incubation, C_t is the total ASA and SA concentrations in the liposome suspension, and C is the ASA and SA concentrations in the sample supernatant after time, t.

4.3. Results and Discussion

4.3.1. Preparation of Microencapsulated Liposomes

Microcapsules prepared by coacervation usually employ an organic disperse phase of an oil-in-water emulsion to contain the material to be encapsulated. In the present study MLVs constituted the disperse phase on the surfaces of which the coacervate of acacia and gelatin were deposited. Evidence of this is seen in Figures 4.1 and 4.2. In Fig. 4.1 electron micrographs of a negatively-stained DPPC liposome is shown before (A) and after (B) being microencapsulated by a shell of acacia and gelatin coacervate. It indicates that the material on the surface was not liposomal but presumably the acacia-gelatin polymer. Fig. 4.2 provides additional evidence from the ultrathin section technique that the multilamellar structure of the liposomes was retained after microencapsulation. Particle size data also suggest that microencapsulation has significantly increased the size of the liposomes at 3.0% and 5.0% acacia-gelatin concentration (F-test, $p=0.05$) and that this was due to the formation of the polymeric shell surrounding the liposomes (Table 4.1). At 5 percent acacia-

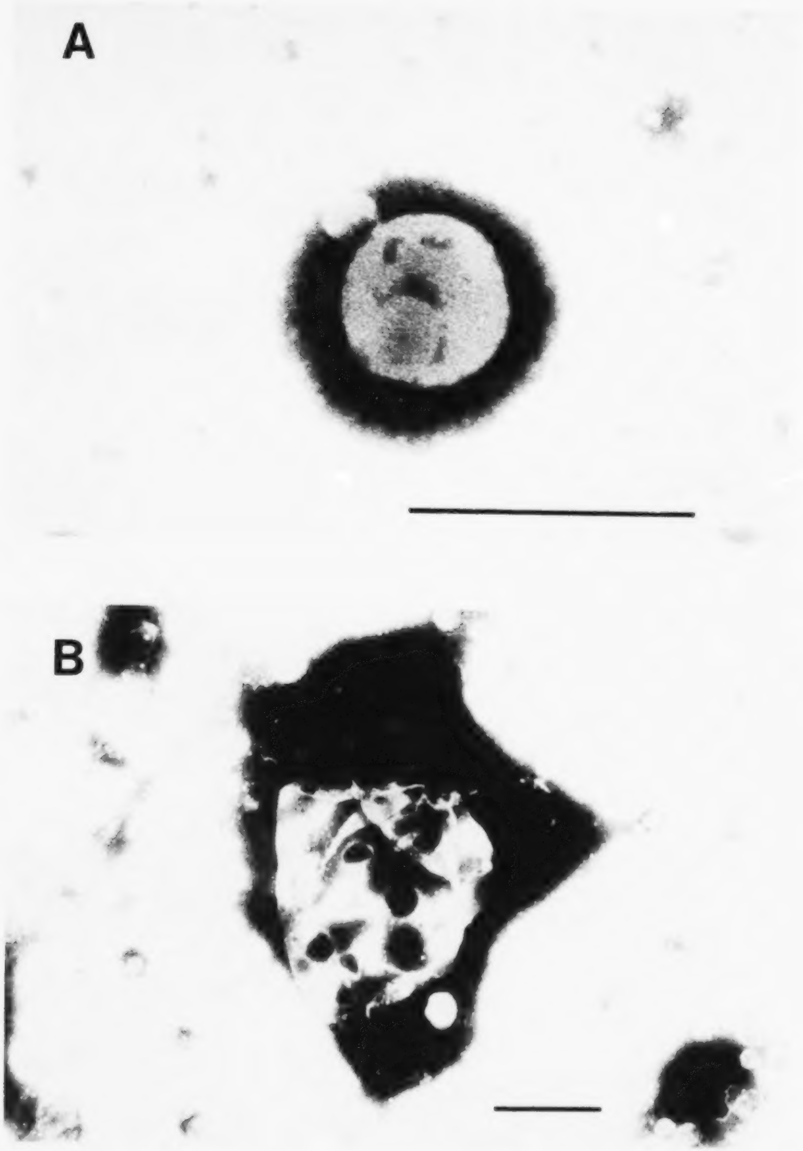

Fig. 4.1. Negative stain electron micrographs showing the microstructures of A) control liposomes; and B) 3% acacia-gelatin-microencapsulated DPPC liposomes. Bar length = 1 μm.

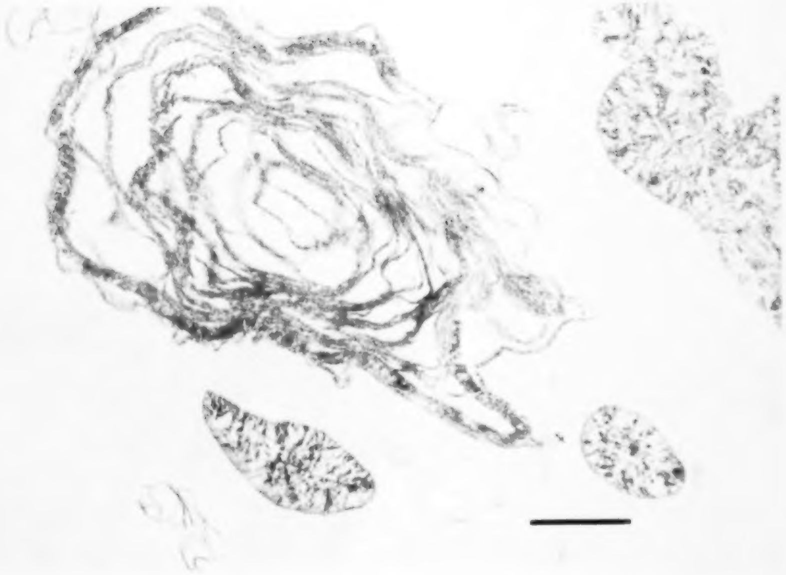

Fig.4.2. Electron micrograph of acacia-gelatin-microencapsulated DPPC liposomes by fixation and thin section technique. Bar length = 1 μm.

Table 4.1. Mean diameter, and size range of acacia-gelatin-microencapsulated liposomes as a function of acacia-gelatin concentration with a hardening time of 1 hr.

Acacia-gelatin Conc. (%)	Average diameter μm (SD)	Diameter range (μm, $p=0.1$)
0	2.76 (0.16)	1.25 ~ 4.69
1.5	2.96 (0.23)	0.92 ~ 5.74
3.0	3.53 (0.13)	1.08 ~ 6.86
5.0	3.50 (0.21)	0.97 ~ 6.98

gelatin, excess coacervate was removed by rinsing or it penetrated the liposome. Also, the particle sizes were not influenced by formaldehyde hardening times of 1 to 4 h.

4.3.2. Evaluation of Liposome Stability

As shown previously, and confirmed here, turbidity changes occurring in the liposome preparations are proportional to the number of liposomes, and in this case microencapsulated liposomes, after irradiation at 400 nm (*Regen et al., 1980*). The sodium cholate reduces the number of liposomes by solubilization without itself interfering with the turbidity measurement. Zero turbidity resulted after complete disruption of liposomes. The relative stabilities of microencapsulated and control liposomes at pH 5.6 and 7.0 are depicted in Fig. 4.3. The liposomes are shown to be fairly stable in sodium cholate solutions at pH 7.0, hence no difference could be seen between microencapsulated and control liposomes. In contrast, control liposomes rapidly disintegrated upon addition of cholate solutions at pH 5.6 whereas microencapsulated liposomes were significantly resistant (F-test, $p=0.05$), only about 30 percent reduction in the turbidity occurring with concentrations as high as 8 mM sodium cholate. At pH 7.0 negligible amounts of cholic acid exist ($pK_a = 5$) (*Roda et al., 1990*) compared to pH 5.6 (20 percent unionized) suggesting that the incidence of mixed micelle formation of phospholipid and cholate over this concentration range is attributed primarily to cholic acid levels in the system (*Nagata et al., 1990*). The detergent

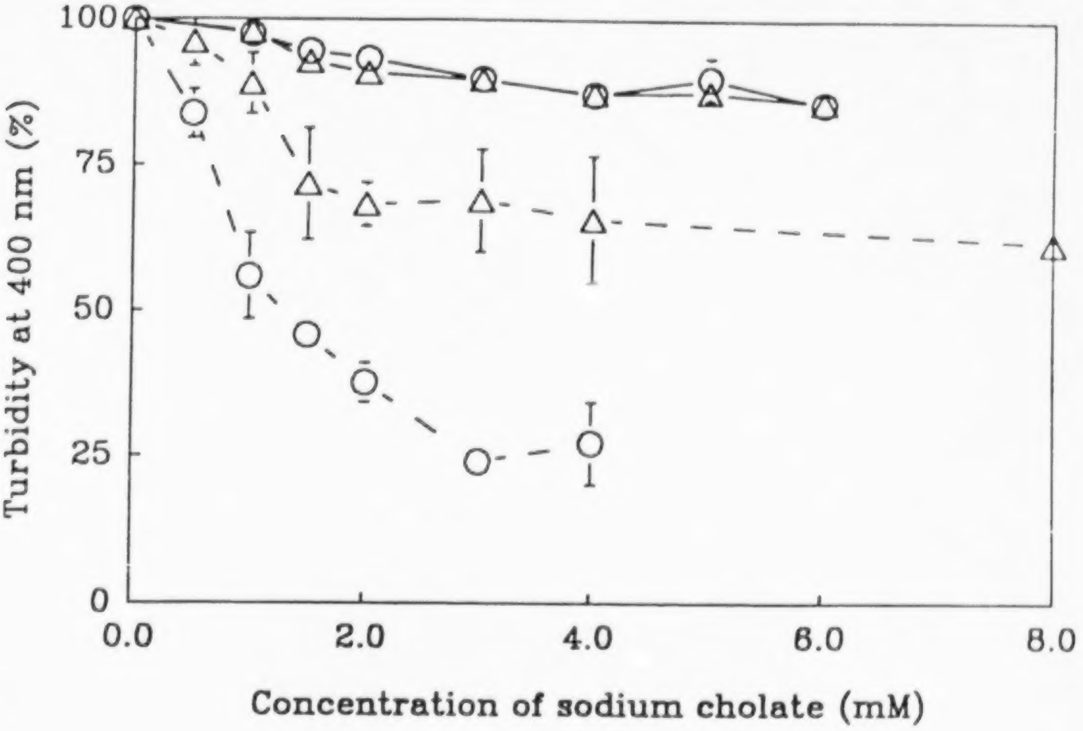

Fig. 4.3. Physical stability of liposomes at 23°C as a function of pH. ○, control liposomes; Δ, 3.0% acacia-gelatin-microencapsulated DPPC liposomes at pH 7.0 (—), and pH 5.6 (---).

activity of sodium cholate has been reported to be greater at pH 5.6 due to protonation, which can be near the pH in the duodenum where bile salts are excreted into the GIT (*Notari, 1987*), than at pH 7.0 (*Nagata et al., 1988 & 1990*). Hence, microencapsulated liposomes could have a distinct advantage in this situation.

When the concentration of acacia-gelatin is increased the coating thickness and, hence, the stability in sodium cholate solution might be expected to increase accordingly. Fig. 4.4 shows these results at pH 5.6 and 23°C. Not unexpectedly, there exists a fairly narrow range of concentration over which microencapsulation is able to occur and the wall thickness reaches a maximum. Thus, at 1.5 percent acacia-gelatin no increase in stability of the liposomes was found, indicating the presence of insufficient polymer coacervate to result in the formation of a uniform layer around the liposomes. At the other extreme, 5 percent acacia-gelatin at a hardening time of 1 h yielded microcapsule wall properties that were more susceptible to cholate than at 3 percent which yielded the greatest stability. Since the particle size also did not increase at 5 percent it appears that the excess polymer penetrated the liposomal bilayers causing a reduction in the overall integrity of the liposomes leading to a lower energy requirement of the cholate to mix with the phospholipid molecules and cause partial solubilization, *i.e.* reduction in turbidity compared to liposomes microencapsulated with 3 percent acacia-gelatin. A similar observation has been made previously with other polysaccharide-coated liposomes (*O'Connor et al., 1985*). Another factor affecting

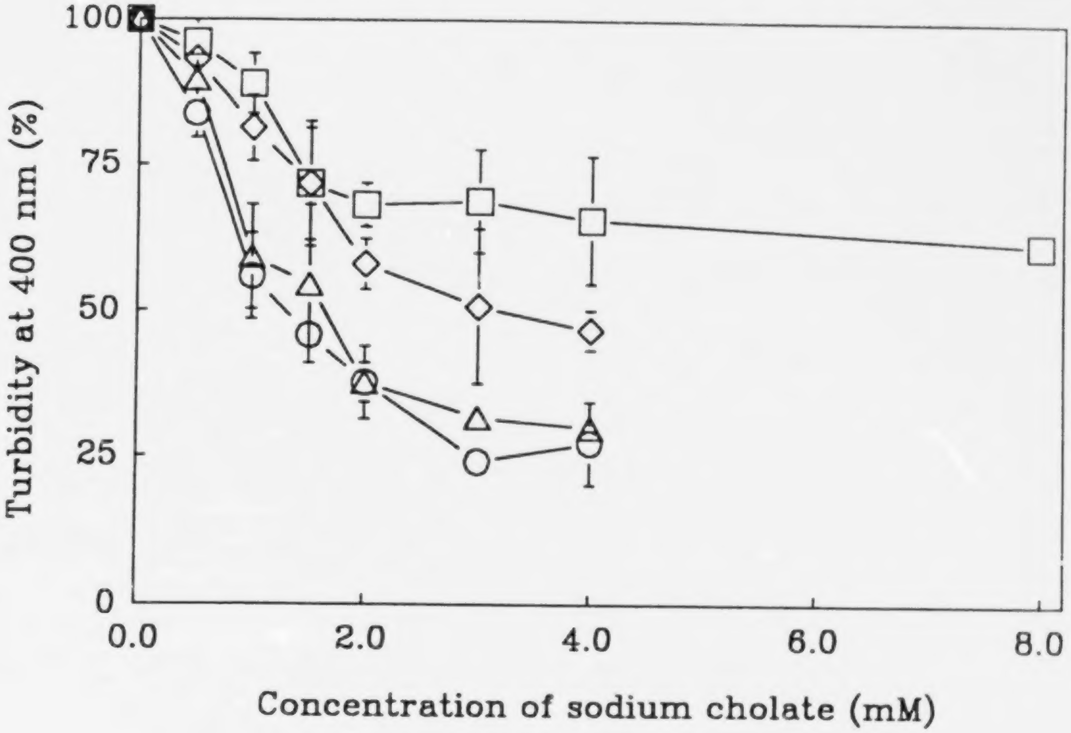

Fig. 4.4. The effect of acacia-gelatin concentration on the physical stability of microencapsulated DPPC liposomes in pH 5.6 acetate buffer at 23°C. ○ ,control liposomes; microencapsulated liposomes with an acacia-gelatin concentration of, Δ, 1.5%; □ , 3.0%; and ◇ , 5.0%.

the formation and coherent structure of the microcapsule wall is the formaldehyde hardening time. While it might be expected that a minimum hardening time would be required it is apparent in Fig. 4.5 that 1 h was sufficient before harvesting the microcapsules and 2 h made no significant difference. On the other hand, there appeared to be a deterioration in the microcapsule wall if it was left to harden for 4 h since the turbidity decreased significantly at 3 mM cholate compared to 1 or 2 h hardening times. Although the reason for this is unclear the prolonged presence of the formaldehyde may have influenced the integrity of the microcapsule wall through denaturation and cracking (*Deasy, 1984*).

4.3.3. Effect of ASA Release

The encapsulation efficiency of ASA in liposomes was determined to be 8.3±1.3% (n = 3) and was unchanged after the microencapsulation process. The influence of acacia-gelatin concentration and temperature on the release of ASA from microencapsulated liposomes is shown in Figures 4.6 and 4.7. There is a dramatic difference in the amount of ASA released within the first 20 min at room temperature and 37°C, less than 5 percent and approximately 90 percent, respectively. Nevertheless, concentrations of acacia-gelatin reduced this happening. Even at 1.5 percent acacia-gelatin some reduction in the release of ASA was observed, although at this concentration it had been shown that no increase in particle size or decrease in stability of the liposomes occurred. However, at 3.0 or 5.0 percent the initial release of ASA was diminished the most

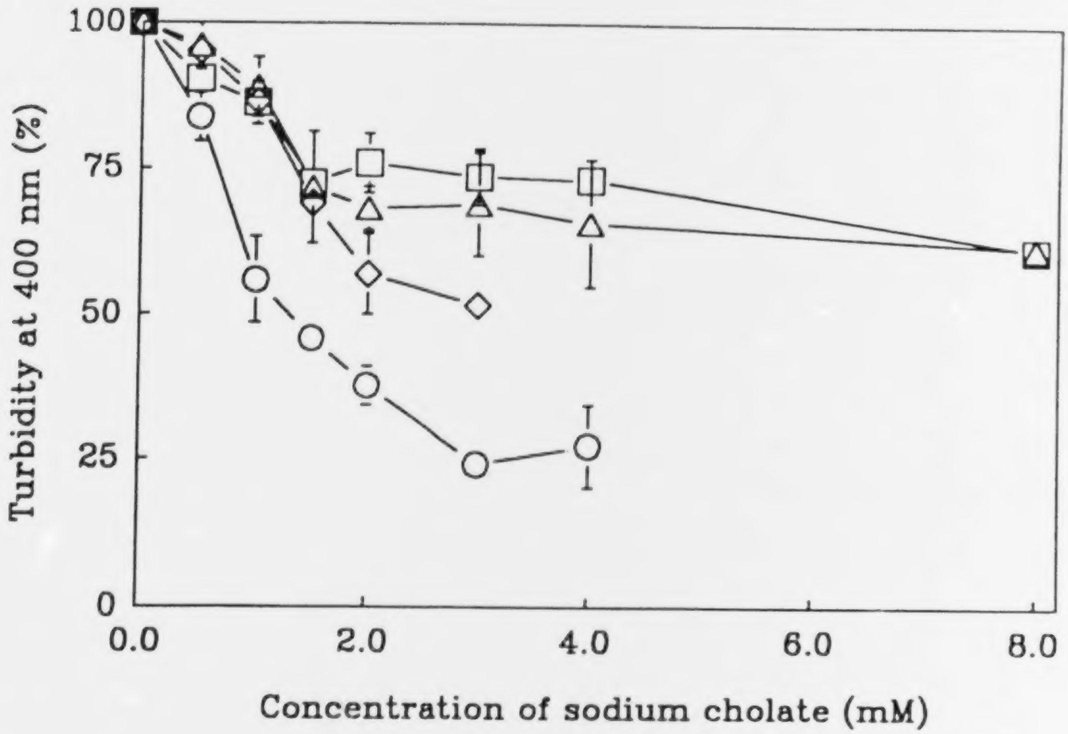

Fig. 4.5. The effect of formaldehyde hardening time on the physical stability of acacia-gelatin-microencapsulated DPPC liposomes in pH 5.6 acetate buffer at 23°C. ○, control liposomes; 3.0% acacia-gelatin-microencapsulated liposomes with a hardening time of, Δ, 1 hr; □, 2 hr; and ◇, 4 hr.

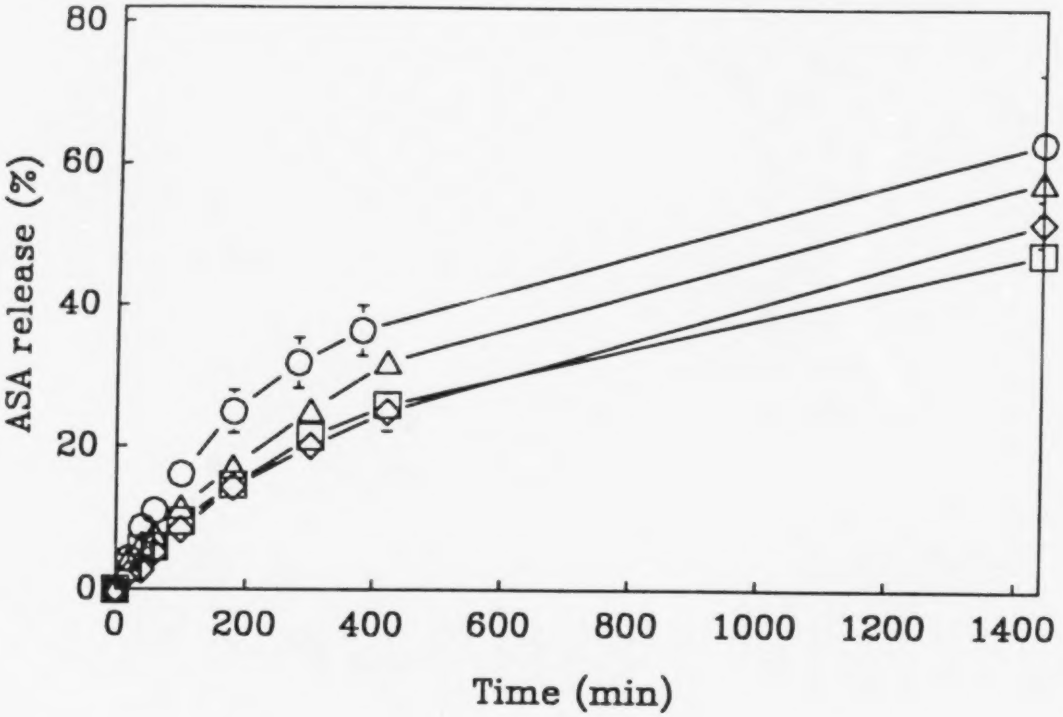

Fig.4.6. The effect of acacia-gelatin concentration on the release of ASA from microencapsulated DPPC liposomes in pH 5.6 acetate buffer at 23°C. ○, control liposomes; microencapsulated liposomes with an acacia-gelatin concentration of, Δ, 1.5%; □ , 3.0%; and ◇ , 5.0%.

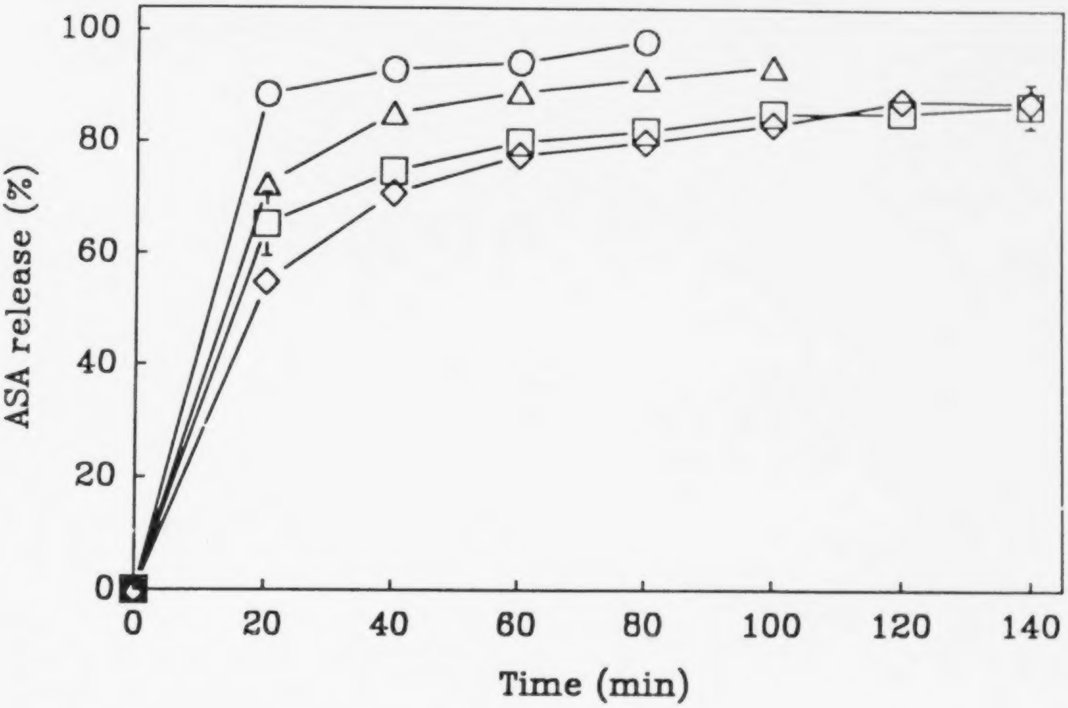

Fig. 4.7. The effect of acacia-gelatin concentration on the release of ASA from microencapsulated DPPC liposomes in pH 5.6 acetate buffer at 37°C. ○, control liposomes; microencapsulated liposomes with an acacia-gelatin concentration of, Δ, 1.5%; □, 3.0%; and ◇, 5.0%.

and was sustained with kinetics which were approximately equal for all formulations, including the control liposomes (F-test, $p=0.05$). These results suggest a matrix-type release at 23°C and Fig. 4.8 confirms adherence to the equation (*Higuchi, 1961*),

$$Q = K\,t^{1/2}$$

where the amount of ASA released, Q, is a function of $t^{1/2}$ and the linear relationship of Q versus $t^{1/2}$ has a correlation coefficient, r, of 0.987. Although similar kinetics are followed at 37°C, the release is dominated by a rapid phase in the first 20 min, probably due to slight osmotic effects due to the encapsulated ASA but mainly due to the change in temperature from R.T. to 37°C. Compared to control liposomes, the slower release of ASA from microencapsulated liposomes is due to the necessity of diffusion through two barriers, the liposomal bilayers then the microcapsule wall. This mechanism has even been suggested as a means of delivering a drug by pulsed release (*Kibat et al., 1990*). A hardening time in excess of 2 h was also found to be detrimental for sustained release of ASA (Fig. 4.9) consistent with the decreased stabilities found for microcapsules subjected to extended hardening times.

4.4. Summary

The microencapsulation of DPPC liposomes using acacia-gelatin and a coacervation technique yielded particles ranging in size from about 1 μm to 7 μm in diameter. The stability of the microcapsules in sodium cholate solutions was

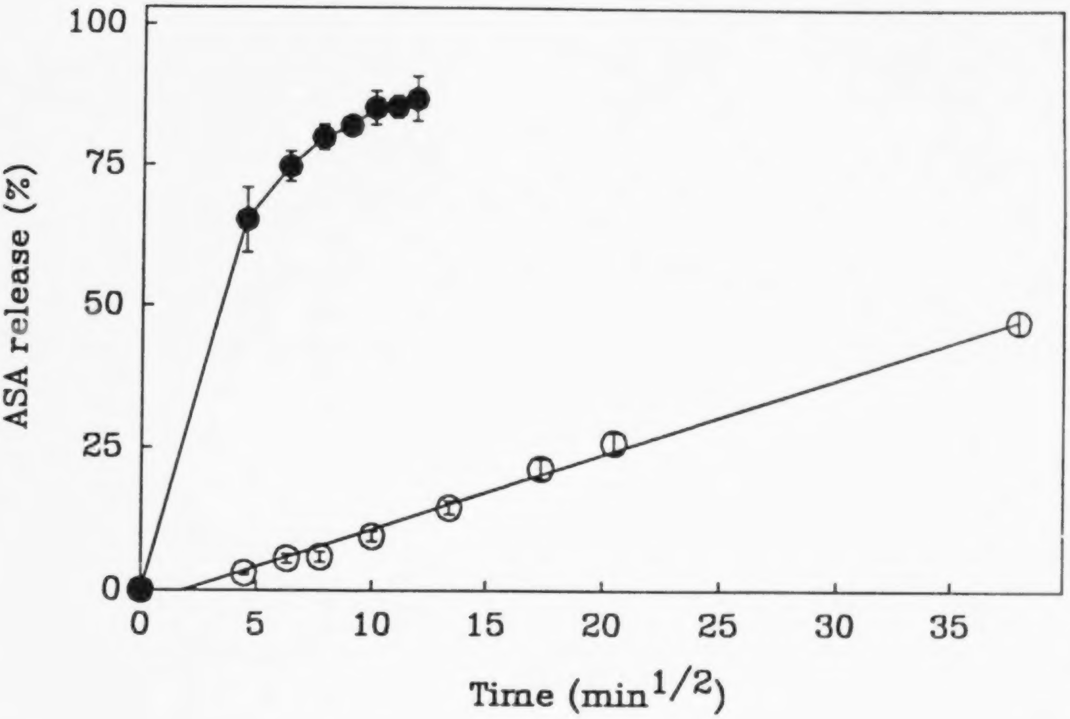

Fig. 4.8. Release of ASA as a function of $t^{1/2}$ from 3% acacia-gelatin-microencapsulated DPPC liposomes at 23°C, ○ , and 37°C, ● at pH 5.6.

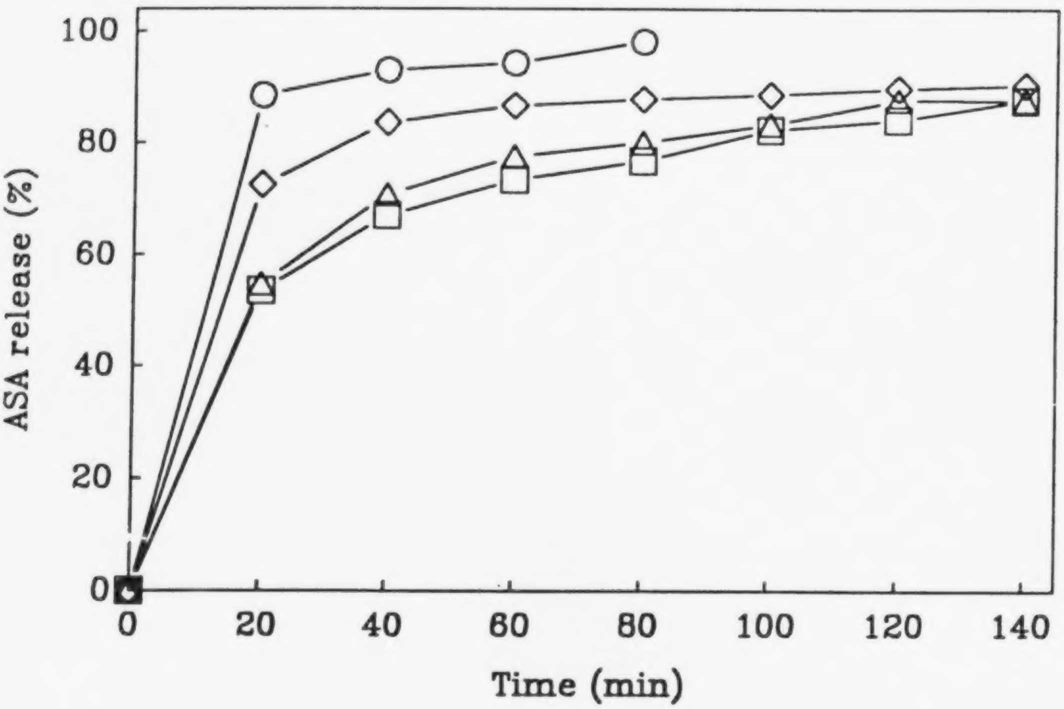

Fig. 4.9. The effect of formaldehyde hardening time on the release of ASA from acacia-gelatin-microencapsulated DPPC liposomes in pH 5.6 acetate buffer at 37°C. ○ , control liposomes; 3.0% acacia-gelatin-microencapsulated liposomes with a hardening time of, Δ , 1 hr; □, 2 hr; and ◇ , 4 hr.

much greater than the corresponding liposomes. The encapsulation efficiency of ASA was unaltered by the microencapsulation process. Optimum composition and conditions for stability as well as release of ASA were 3.0 percent acacia-gelatin and 1 h of hardening time with formaldehyde. The kinetics of ASA release conformed to a model of transport across two diffusion barriers, namely, the lipid bilayers and the microcapsule wall of which the rate-determining step after the initial release phase was diffusion from the liposomes. A marked increase in the amount released at 37°C (cf. 23°C) but without change in the release pattern substantiates this conclusion. These properties of microencapsulated liposomes suggest their possible use as oral drug delivery systems.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

5.1. General Discussion

Liposomes have been extensively studied as drug delivery systems by different routes of administration, such as intravenous (*Alving et al., 1978; New et al., 1978; Gabizon et al., 1989*), intrapulmonary (*Mihalko et al., 1988*), intraperitoneal (*Markman et al., 1986*), intramuscular (*Eppstein and Felgner, 1988*), subcutaneous (*Kim and Howell, 1987*), ocular (*Guo et al., 1988*), topical (*Mezei, 1988*), and oral (*Patel et al., 1982*). The oral route is the most convenient, versatile, and patient acceptable. However, this route has not been successful because of liposome instability in the GIT, especially due to the detergent action of bile salts. Many attempts have been made to overcome these problems, but little progress has been made so far.

In the early stages of development, improvements in liposome stability concentrated on alterations of liposome composition. Liposomes of DSPC:CHOL (2:1) which were believed to be the most stable were unable to survive in the GIT (*Chiang, 1986*). An alternative method was to use modified liposomes such as polymerized liposomes (*Regen, 1987*) or polymer-coated liposomes (*Sunamoto et al., 1987*). Both approaches have been shown to stabilize liposomes. In comparison, polymerized liposomes significantly changed the bilayer characteristics of liposomes, whereas polymer-coated liposomes appeared to retain their bilayer properties similar to uncoated liposomes, but confirmation of this has not been made. The objectives of this study were to develop polymer-coated liposome systems, test their stabilities against the detergent effect of bile salt solutions, and quantitate the kinetics of release of entrapped solute in order to increase the possibilities of developing effective liposomes for oral administration.

In principle, liposomes can be coated with polymers via hydrophilic adsorption (*Seki and Tirrell, 1984*), ionic forces (salt formation) (*Aliev et al., 1984*), or due to penetration of the bilayers by hydrophobic anchors attached to the polymer (*Sunamoto et al., 1987*). In practice, one or more mechanisms can be operative for a particular polymer. The application of poly(4-VP) (*Aliev et al., 1984*) and CM-chitin (*Izawa et al., 1986*) to coat liposomes and a coacervate of nylon-gelatin-acacia (*Nixon and Yeung, 1989*) to microencapsulate liposomes have been reported. However, none of these have been developed as oral drug delivery systems.

Perhaps degradation of ASA was important, and the stability of ASA was not the objectives of this thesis, because this has been dealt by others (*Habib, 1987*).

In the first instance, the ability to prepare poly(4-VP)-coated liposomes of DMPC and containing varying amounts of the negatively-charged amphiphile, DCP, was investigated. This was in contrast to the use of only DCP liposomes used by Aliev et al. (1984) to demonstrate the principle. Furthermore, the effects of polymerization on the encapsulation of ASA as a water-soluble solute, and on the resultant ASA release kinetics were explored. The outcome of these studies were very encouraging. Not only could poly(4-VP)-coated liposomes be prepared with an optimum DMPC:DCP, 1:1 mole ratio, but it was shown by TEM that the lamellar structure of the liposomes was retained and that the coating process had no influence on the encapsulation efficiency of ASA. The release kinetics further indicated that poly(4-VP)-coated liposomes released a smaller fraction of ASA during the initial rapid phase than the corresponding uncoated liposomes. The appearent slow release during the second phase was similar for the coated and uncoated liposomes which meant that the period over which ASA could be released was extended when using the poly(4-VP)-coated liposomes. Although the potential toxicity of poly(4-VP) (even after total removal of residual 4-VP monomer) is unknown and has not been tested, the principle of formulating a stable, sustained release phospholipid liposome by coating with a polymer was demonstrated. Most importantly, the stability of poly(4-VP)-coated liposomes was found to be considerably greater in sodium cholate solutions than the uncoated liposomes.

Another approach was to determine the extent to which a polysaccharide could stabilize liposomes and alter the release kinetics of ASA. CM-chitin chosen for this purpose was incorporated in two ways - addition to preformed liposomes or addition via the aqueous phase during liposome preparation, *i.e.* during hydration of the lipid film. The coating efficiency of DPPC liposomes varied with CM-chitin concentration but was optimum at 1 percent. Regardless of which procedure was used in preparing the polymer-coated liposomes they were spherical and appeared as uniform spheres by optical microscopic inspection indicating that polymer resided at the surfaces of the liposomes. Again, it was demonstrated that the polymer coating process did not influence the encapsulation efficiency of ASA. The release kinetics further indicated that CM-chitin-coated liposomes reduced the release rate of entrapped ASA compared to uncoated liposomes, the magnitude of which was a function of the amount of polymer added. In comparison to poly(4-VP)-coated liposomes, the decreased release was more pronounced. Similar to poly(4-VP)-coated liposomes, the physical stability of CM-chitin-coated DPPC liposomes was greater than uncoated liposomes by several orders of magnitude at the optimum 1% CM-chitin concentration added during liposome formation. At a lower CM-chitin concentration (0.5%), the stability against solubilization by detergent was less, presumbly because of a thinner polymer coating at the surfaces, although this could not be determined directly. At a 2 percent CM-chitin concentration, the stability was not any better than at 1 percent indicating a limit in the properties of the polymer film resistant to the action of the bile salt. Furthermore, *O'Connor et al. (1985)* have shown that linear polysaccharide-coated liposomes, which includes CM-chitin, were more stable in serum than branched polysacchride-coated liposomes. It was shown that turbidity measurements, as an indication of the degree of liposome solubilization, could not only be used to provide stability information but also be applied in the quantitation of the CM-chitin associated with the liposomes.

A slightly different approach in preparing a polymer-coated liposome was to employ a microencapsulation method which did not involve organic solvent as is usually the case using this technique. This was also in contrast to the microencapsulated liposomes prepared by Nixon (*Nixon and Yeung, 1989; Yeung and Nixon, 1988*) from an emulsion of cyclohexane/chloroform in aqueous liposome suspension. *Kabat et al. (1990)* prepared microencapsulated liposomes in which each microcapsule contained multiple liposomes whereas close examination of the acacia-gelatin microcapsules by TEM in this study revealed individually microencapsulated liposomes of which the lamellar bilayer structure was preserved. As with poly(4-VP)- or CM-chitin-coated liposomes, the microencapsulation process did not alter the encapsulation efficiency and the microencapsulated liposomes released ASA at a slower rate than uncoated liposomes. These systems possessed stability and release rate characteristics intermediate between poly(4-VP)- and CM-chitin-coated liposomes although more similar to the latter systems.

Thus, three polymers have been successfully used to coat/microencapsulate on the surface of the liposomes without any apparent alteration of the bilayer integrity at the optimum concentrations or changing the ASA encapsulation efficiency. Furthermore, all polymer-coated or microencapsulated liposomes decreased ASA release by different magnitudes and the decrease was in the order of CM-chitin-coated > acacia-gelatin-microencapsulated > poly(4-VP)-coated liposomes. This indicates that different release rates can be achieved by selection of different polymer coatings and changes in polymer concentrations. It can also be predicted that different hydrophobicity of the drug as well as its molecular weight will also significantly influence drug release. One might eventually envision formulations which control drug release at pre-determined desired rates. Most importantly, the stability of polymer-coated/microencapsulated liposomes were demonstrated to be considerably more stable in sodium cholate solutions than control liposomes. It is anticipated that these stabilized liposomes can protect liposomes from destruction in the GIT when given orally. The appropriate level of stability can be achieved again by selection of suitable polymer systems. This characteristic plus the controlled release of drug are the basic requirements to be achieved by polymer coating or microencapsualtion of liposomes intended for oral administration.

Potential applications could include macromolecules such as insulin or other proteins, hormones, polypeptides in which oral administration of polymer-coated/microencapsulated liposomes would result in an increased bioavailability by protecting the drug from the destructive elements of the GI fluid, controlling the drug release rates and possibly facilitating drug absorption. Approaches to the latter might involve increasing the residence times of formulations by virtue of a mucoadhesive or bioadhesive characteristic of the surfaces of polymer-coated liposomes. This may also entail attachment of a site specific determinant to the liposome surfaces such as tomato lectin, which has been proposed as a binding ligand to intestinal mucosa (*Woodley and Naisbett, 1988; Naisbett and Woodley, 1989*).

5.2. Conclusions

1. Liposomes can be coated with poly(4-VP) or CM-chitin and microencapsulated with acacia-gelatin. The coating procedures neither alter the integrity of liposomes nor influence the ASA encapsulation. The optimum concentrations for polymer coatings were 8.5 mM 4-VP, 1% CM-chitin, and 3% acacia-gelatin.
2. ASA release from poly(4-VP)-coated liposomes was significantly reduced compared with control liposomes. However, the reduction in extent of release of ASA was most pronounced from CM-chitin-coated liposomes followed by acacia-gelatin-microencapsulated liposomes.

3. The physical stabilities of polymer-coated liposomes in sodium cholate solutions were greatly increased compared to control liposomes. Again, CM-chitin-coated liposomes were found to be the most stable formulations followed by acacia-gelatin-microencapsulated liposomes then poly(4-VP)-coated liposomes.
4. Two of the most important properties of an oral liposome drug delivery system has been shown to exist by employing selective polymers to coat or encapsulate the liposomes. These are increased stability in a typical bile salt solution and slower release of entrapped ASA, representing a small water-soluble permeant, compared to the corresponding uncoated liposomes. Further testing both *in vitro* and *in vivo* is therefore warranted.

5.3. Future Work

Future work could involve improvements in the poly(4-VP) type coated liposomes. For example, other vinylpyridinium analogues such as 1,2-dimethyl-5-vinylpyridinium methyl sulfate could be used (*Kabanov et al., 1968*). Alternately, other positively-charged monomers, such as choline methacrylate, bis(2-methacryloyloxyethyl)dimethylammonium bromide (*Ringsdorf and Schlarb, 1988*) could be used instead of a vinylpyridium monomer. More recently, it has been suggested that polysaccharides such as pullulan, mannan, dextran or amylopectin derivatized with cholesterol to anchor the polysaccharide to the liposomal surfaces might yield liposomes of improved stability and functionality. These have the advantages of low toxicity, biodegradability and low cost. However, ultimately, a better understanding of the mechanisms involved and requirements for particulate delivery of drug across the GIT is required in order to achieve the successes warranted by these novel approaches to improved drug therapies by the oral route.

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APPENDIX

CALCULATION OF IONIZATIONS OF 4-VP AND DCP

The pK_a of an organic base can be quantitatively calculated by the Hammet equation (*Perrin, 1980*):

$$\log(k/k^o) = (\Sigma\sigma)\,\rho \qquad (1)$$

Here k and k^o are the ionization constants for the substituted and the unsubstituted compounds, respectively. σ is a constant for the particular substituent and ρ is constant for a particular equilibrium reaction. From (1),

$$pK_a = pK_a^{\circ} - (\Sigma\sigma)\,\rho \qquad (2)$$

The pK_a° of pyridine is 5.25, ρ is 5.9, and σ is -0.08 (*Clark and Perrin, 1964; Perrin, 1980*). Hence, for 4-VP

$$pK_a = 5.25 - (-0.08) \times 5.9 = 5.72$$

The pK_a of DCP has been reported to be 4.5 (*Maitani et al., 1990*), which was obtained by a ζ-potential method.

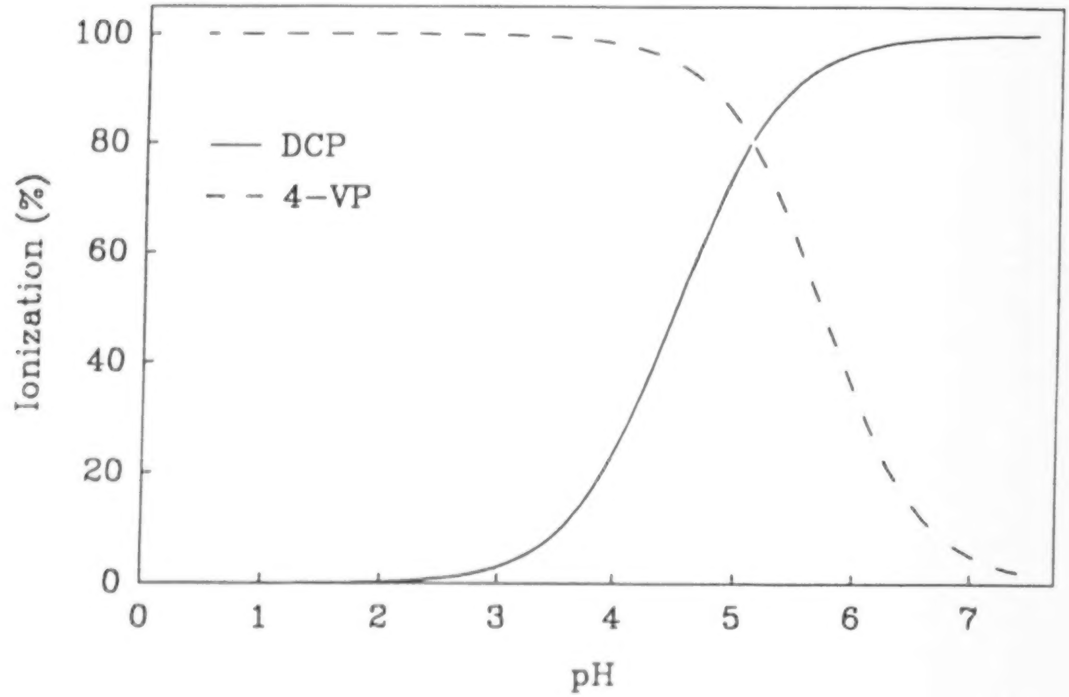
Calculations of percentage ionization at different pHs are based on the following equations (*Martin et al., 1983*):

$$\text{Ionization \% of DCP} = 100 \times 10^{(pH-4.5)}/(1 + 10^{(pH-4.5)}) \qquad (3)$$

$$\text{Ionization \% of 4-VP} = 100 \times 10^{(5.72-pH)}/(1 + 10^{(5.72-pH)}) \qquad (4)$$

Since the pK_a is also a function of the solvent used, the predicted pK_a can only be an approximate value. However, this is sufficiently accurate in making predictions of degree of ionization of 4-VP and DCP.

pH versus percent ionization of both DCP and 4-VP is illustrated as follows.



Percentage ionizations of DCP and 4-VP as a function of pH.

The percent ionizations at selected pHs calculated from equations 3 and 4 are illustrated as follows:

pH	Percentage ionization	
	DCP	4-VP
1.0	0	100
2.5	1	100
4.0	24	98
7.0	100	5

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